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XXIX CICLO

Effect of maternal separation on ethanol drinking and acute stress: involvement of ethanol consumption during pregnancy

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<u>Introduction</u>

Stress

Over the years, the definition of stress has changed. Now the most common definition regards an "oppressive situation or concern caused by problems in the life of an individual". Although, the term stress assumes a negative connotation in everyday language, as a symptom of physical and/or psychological illness, stress is actually able to trigger positive responses in those who experience it, making the individual capable of dealing with situations requiring great concentration and some physical and mental effort.

Stress therefore represents an adaptive response to an infinite variety of external stimuli that affect our bodies every day, and it tends to alter the normal homeostatic balance. The body, subjected to one or more stressful stimuli, initiates a series of physical and neurochemical responses in order to restore the disrupted balance. Stress is described as a concurrent alteration of responses: behavioral (warning, attention and vigilance), vegetative (high blood pressure and cardiovascular activity), sensory and endocrine by the production of hormones such as corticosteroids, which are capable of rendering the body durable and responsive.

Several lines of evidence show that exposure to stressful stimuli, arising from family, social and work environments, that the body is unable to fight, make the individual vulnerable to the development of physical and psychological pathologies such as depression and anxiety.

The term stress was introduced for the first time in medicine by Hungarian scientist Hans Selye in 1936 (***Seyle, 1936***), who carried out studies on stress while trying to isolate sex hormones in animals subjected to a stressful stimulus. In these studies, he observed that the

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same biological reaction, characterized by the common state of hyperactivation of the hypothalamic-pituitary-adrenal axis (HPA axis), appeared in all the experimental animals independently of the type of stressor. The same year, he published a paper in the British Journal Nature: "General Adaptation Syndrome" (GAS), also called the "Stress Syndrome". According to his research, GAS is divided into three different phases:

• <u>Alarm phase</u>: the body comes in contact with the stressor, that induces a "fight or flight" response, which leads to the immediate production of hormones such as cortisol and adrenaline to allow an immediate first response. Also, glucose is involved in many mechanism, as well as an increase in blood pressure and respiratory frequency, which allow the body to remain in the alarm condition.

• <u>Resistance phase</u>: the body tries to counteract the negative effects of prolonged stress, producing a release of endocrine/hormonal responses with the involvement of the adrenal glands.

• <u>Exhaustion phase</u>: if the stressor persists, the individual may become overwhelmed, producing unpleasant permanent effects on mental and/or somatic structures.

The activities of the various systems of the body and the brain needs to be coordinated in order to develop an adaptive response to challenge (fight or flight response). In the periphery, it results in an increased blood flow to the muscles and the heart, while the immune system is inhibited. In the brain, all cognitive processes should be directed to an adaptive response to a potentially dangerous situation; all processes that are not immediately required will be reduced or inhibited. Most of these actions due to stress in the periphery occurred through the autonomic nervous system, which involves adrenaline and noradrenaline. The endocrine mechanisms involved in the stress response are under the control of Hypothalamic-Pituitary-Adrenal axis (HPA axis). During a stress event the hypothalamus is the first to be involved in

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the adrenal gland activation, regulating the secretion of hormones (among which the most important is cortisol in humans and corticosterone (CTS) in rodents) involved in these responses (Figure 1)
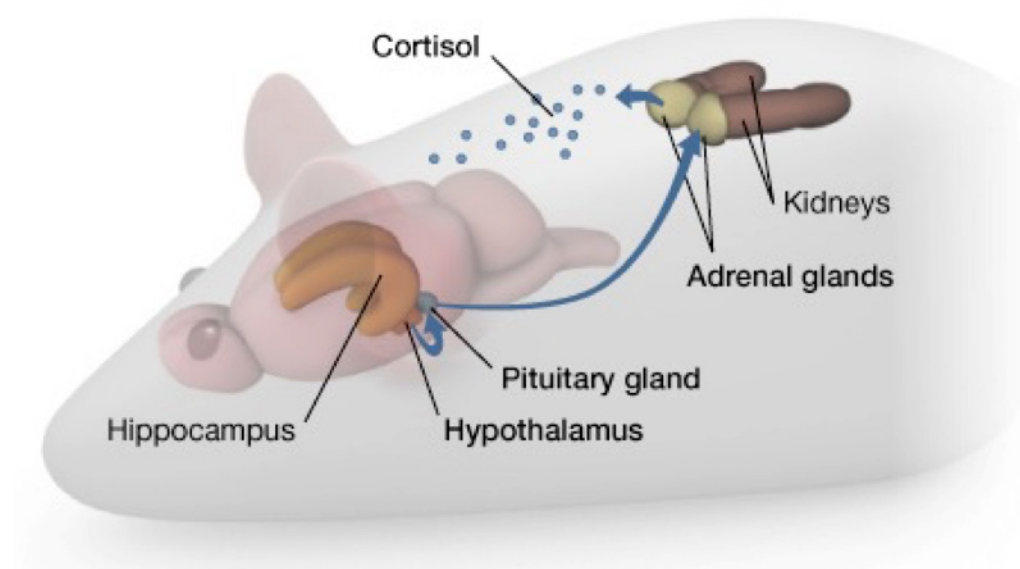

Figure 1. Schematic rendering of the rat HPA axis

In mammals, gestation and later childhood are two important periods of high plasticity in the central nervous system (***Greenough et al., 1987; O'Leary et al., 1995***). Evidence shows that stressful situations, either physical or psychological, that occur during the neo/perinatal period, can negatively affect brain development and subsequent behavior in adulthood (***Anisman et al., 1998; Weiss and Feldon, 2001***). Also, in the human, negative experiences during childhood and adolescence may increase the probability of psychiatric diseases occurring, such as schizophrenia, depression or anxiety disorders (***Lewis and Miller, 1990; Weinberger, 1987; Heim and Nemeroff, 2001***). The exposure to specific stimuli during critical periods of development can permanently alter tissue organization and gene expression. The gestational period and then prenatal development are characterized by environmental

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stimuli, during which the body is particularly vulnerable. Several studies, in recent decades, have shown that stress during pregnancy can lead to fetal functional alterations such as gene dysregulation, destruction of neurons and synapses, inhibition of the maturation of dendrites (*Lou et all., 1994; Zheng et al., 2016*) and inappropriate development of the corpus callosum (*Lemaire et all., 2000*). According to Van den Bergh and colleagues (2005), there are two main explanations of how the emotional state of the mother affects the fetus. The first is the alteration of the uterine blood flow, the second is the effect of hormones through the placenta. High concentrations of stress hormones during gestation may have effects on the development of the fetal nervous system inducing possible changes in the prefrontal cortex which is involved in the regulation of cognitive and emotional aspects. The nature and severity of effects are influenced by the degree of exposure and the stage of pregnancy. Moreover, maternal exposure to stressful stimuli during the third trimester, a period of organ development, can lead to serious consequences in cognitive, cardiac, visual, auditory, reproductive and digestive function in the child. Exposure to stress during the second and third trimester may result in low birth weight, skeletal and hearing defects (*Talge et al., 2007*).

Childhood is the period from birth to pre-adolescence. At the age of six, the size of child's brain is larger than 90-95% compared to an adult one. Humans reach their maximum brain density between the third and sixth month of pregnancy, the culmination of the explosive prenatal neuronal growth. During the last months of gestation, the brain undergoes a dramatic reduction in its volume, and unnecessary brain cells are eliminated. At birth, the human brain is far from fully formed. Although the most intense development occurs in childhood, the brain still continues to form throughout life. The study by Van den Bergh and colleagues (2007) was able to show how stressful situations experienced between the twelfth and twenty-

second week of gestation are associated with abnormal HPA axis (measured through the salivary cortisol levels throughout the day) in offspring that results in symptoms of depression in adolescence. During childhood, the process known as "synaptic pruning" continues, in which the brain actually loses the least used neuronal connections, forming other very strong synapses in the most used neuronal circuits (Figure 2).
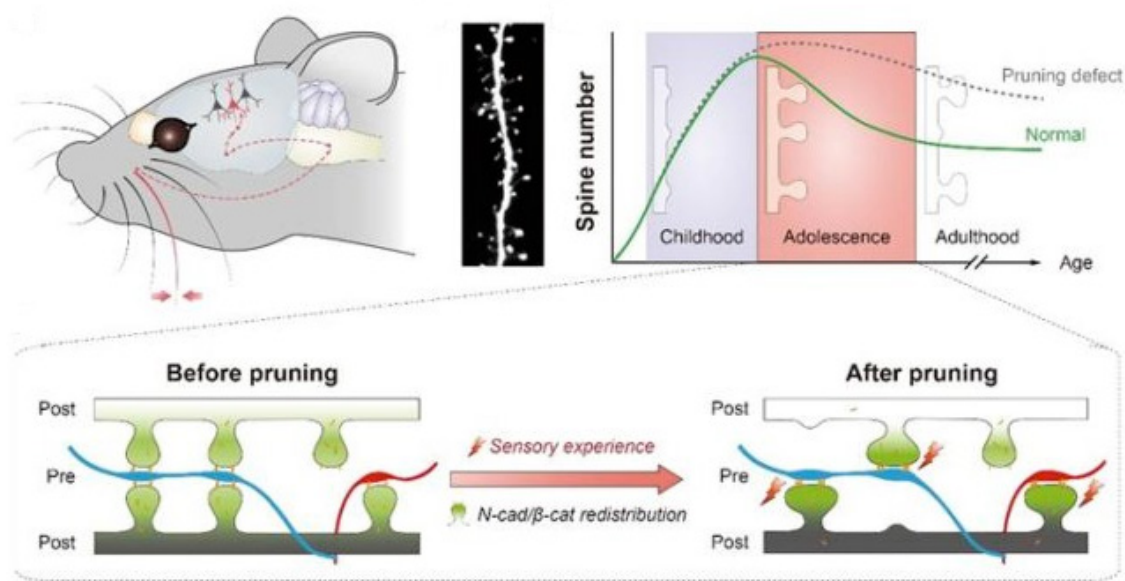

Figure 2. A mouse model of spine pruning. (Credit: Yu Xiang/CAS)

These processes are crucial to the development of the childhood brain. In fact, diseases such as ADHD (Attention-Deficit/Hyperactivity Disorder) and Tourette's syndrome, clinically presenting in preschool, may be related to this brain proliferation period. Although, these diseases have genetic roots, the rapid growth of the brain tissue in early childhood, especially in regions rich in dopamine, can create the basis for the increase in motor and nervous tics that characterize these syndromes.

During childhood, every experience plays a key role in remodeling the brain and this is demonstrated by the fact that babies learn quickly during the first months of life due to visual, auditory, tactile and olfactory stimulation from the environment in which they grow. Therefore, a favorable environment would seem to be a predisposing factor for proper development of the brain.

Negative experiences during this period, such as abuse suffered during childhood or deprivation of parental care, may cause alterations in brain development that last for a lifetime, because they alter both the functionality and the morphology of the brain. There is in fact, evidence of how the brain volume of children who have been neglected by mothers is reduced compared to children who had received more maternal care (**Glaser 2000**). These alterations also appear to be related to the onset of psychopathologies in adulthood, such as schizophrenia, depression and other mood disorders.

Although, there are still many unsolved questions, but literature show that pregnancy and childhood are absolutely two periods of great vulnerability in brain development, and stressful situations experienced in these periods can cause changes that can last for a lifetime.

These hormone responses exert a self-control mechanism that allows the body to maintain their concentrations at the constant physiological level. In fact, once released into the circulation, they are able to act in a retrograde sense at the level of the hippocampus, hypothalamus and pituitary, through a negative feedback mechanism, inhibiting or slowing the production of CRH and ACTH.

Steroid hormones may affect neuronal function through two mechanisms of action:

 • the first mechanism, the genomic type, is characterized by the binding of steroids to intracellular receptors, forming a complex that migrates into the nucleus and then binds to specific DNA sites and changing transcription;
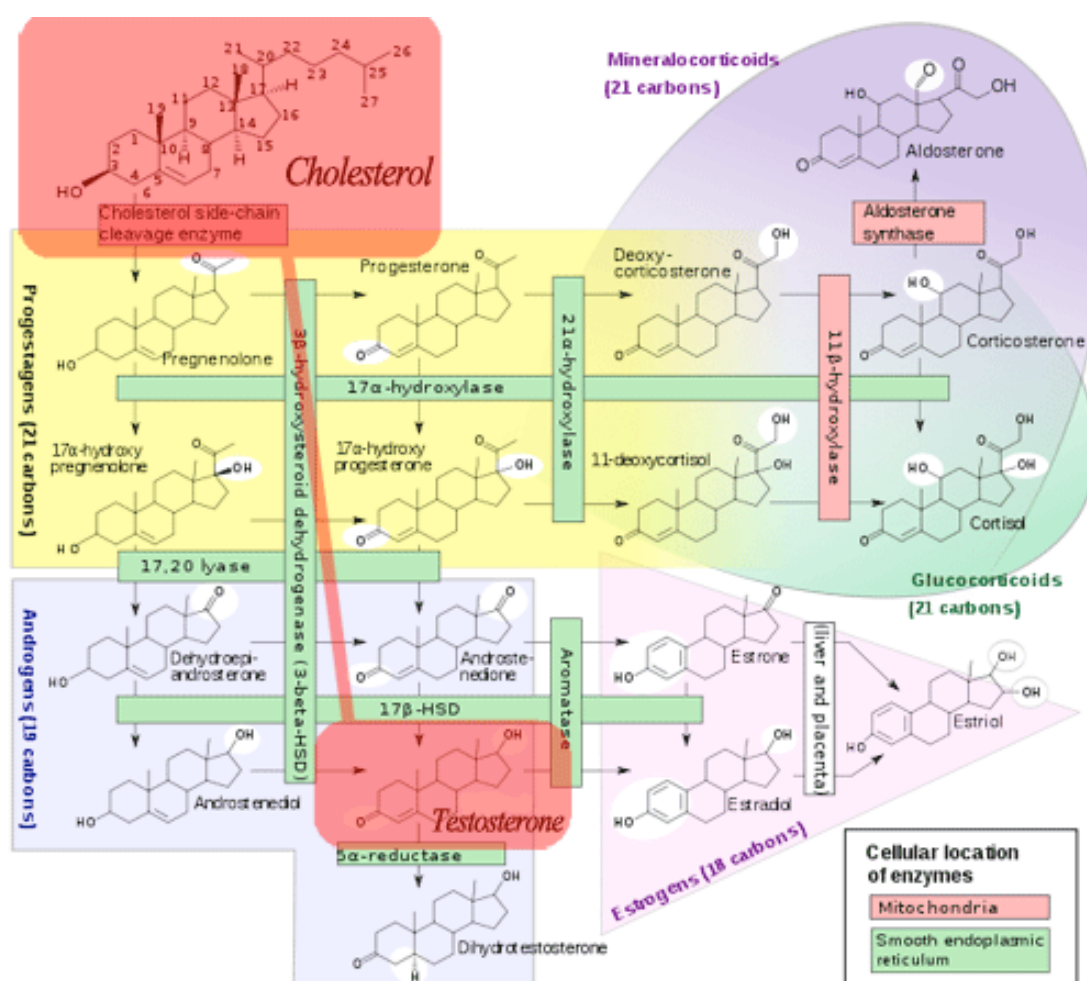
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• the second mechanism consists instead in a non-genomic action, characterized by an immediate onset of effect (from a few seconds to minutes), through which the steroids, by binding to particular membrane receptors, modulates neurotransmitter function. Experimental studies, in fact, have shown that certain nonsteroidal compounds can alter neuronal excitability through interaction with certain receptors for the neurotransmitters (***Majewska et al., 1986; Paul and Purdy, 1992; Lambert et al., 1995***).

Neuroactive steroids and GABAA receptor

Steroid hormones are lipophilic compounds synthesized from cholesterol. They are produced both at the peripheral level, gonads and adrenal gland, and centrally by glia cells and neurons. They exert important effects on the central nervous system. Due to their high lipophilicity, they easily cross the blood-brain barrier and enter the brain. Given that the brain is also able to produce steroid hormones "neurosteroids", hormones directly synthesized in the brain, regardless of peripheral sources. The production of steroids in the adrenal cortex is regulated by the HPA axis, which is the main coordinator of the body's metabolic and behavioral responses to different stressful situations. In fact, the hypothalamus, in response to various signals from higher nerve centers, secretes corticotropin releasing hormone (CRH) into the pituitary portal circulation. Cells in the adenohypophysis cells respond to CRH producing adrenocorticotropic hormone (ACTH), which is secreted into the bloodstream and reaches the adrenal glands, and stimulates the synthesis and secretion of corticosteroids.

The biosynthetic pathway leading to the synthesis of steroids (Figure 4) requires the translocation of cholesterol from the outer mitochondrial membrane to the inner membrane. The transport of cholesterol within the mitochondria is the rate-limiting step of steroid

synthesis. The regulation of this transport is mediated by the activation of "peripheral benzodiazepine receptor" (PBR) (***Woods et al., 1996***). The release of ACTH involves an intracellular increase of cAMP, determining the activation of the DBI (Diazepam Binding Inhibitor) peptide, which activates the PBR, allowing the entry of cholesterol into the mitochondria. Inside the mitochondria, cholesterol, through a reaction catalysed by the enzyme of the side chain cleavage cytochrome P450scc, is converted to pregnenolone (PRE).

Figure 3 Neurosteroid Biosinthesis

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This compound migrates from the mitochondria to the endoplasmic reticulum where, by the action of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HDS), is converted into progesterone (PRO).

PRO can undergo two different metabolic processes:

- by 5α-reductase enzyme action, PRO is converted into 5α-dihydroprogesterone (5α-DHP) which later, through the 3α-hydroxysteroid dehydrogenase (3α-HSD), leads to the synthesis of allopregnanolone (AP)

- by P450c21 enzyme action, PRO is converted into 11-deoxycorticosterone (DOC), and then metabolized to diidrodeossicorticosterone (DHDOC) by 5α-reductase and finally to tetraidrodeossicorticosterone (THDOC).

The two metabolites of PRO, AP and THDOC, founded in vivo and in vitro, are the most potent endogenous positive allosteric modulators of the GABA$_A$ receptor, as they facilitate the interaction of GABA and benzodiazepines with their binding sites and enhance chloride (Cl) currents. The molecular mechanism is dependent upon steroid concentrations: at low concentrations, these compounds facilitate the action of GABA (***Harrison et al., 1987***), while at high concentrations, they are capable of directly opening the channel (***Puia et al., 1990***). Their administration in pharmacological doses determines, in rodents, an anxiolytic, anticonvulsant and sedative-hypnotic effects (***Majewska et al., 1986 and 1992; Harrison et al., 1987; Lambert et al., 1995***). The GABA A receptor is a heteropentamer which is part of the superfamily of transmembrane ion channel receptors. Nineteen types of subunits are known, which have 30-40% homology in their amino acid sequence (***Seeburg et al., 1990***). GABA$_A$ receptors are divided into eight classes named with greek letters: α (six isoforms), β (three isoforms), γ (three isoforms), δ, ε, π, θ, ρ (three isoforms). The existence of different classes of subunits and their respective isoforms (each with a specific brain localization) leads

to the formation of different types of GABA$_A$ receptors that have different pharmacological profiles. In the nervous system of mammals most GABA$_A$ receptors are composed of a combination of two α subunits and two β subunits assembled to form a pentamer with a γ or δ subunit. The GABA$_A$ receptor plays a crucial role in the control of neuronal excitability, and mediates the action of anxiolytic, sedative-hypnotic and anticonvulsant drugs, such as benzodiazepines, barbiturates and imidazopyridines. The evidence that neuroactive steroids are endogenous ligands of the GABA$_A$ receptor, suggests that these hormones may have a crucial physiological role in modulating the threshold of neuronal excitability and thus may control many brain functions. To confirm this, there is evidence that the brain and plasma concentrations of neuroactive steroids undergo, both in humans and rats, marked and temporary changes in several physiological conditions, such as the menstrual cycle (***Genazzani et al., 1998***), pregnancy, childbirth (***Concas et al., 1998***) and menopause (***Genazzani et al., 1998***), or in pathological conditions such as depression (*Romeo et al.1998*). Several studies have also revealed a correlation between stress, neuroactive steroids and GABAergic function (***Strömberg et al., 2008; Pinna G et al., 2000***)

Role of Allopregnanolone in stress

Allopregnanolone (AP), as mentioned above, is one of the most potent positive allosteric modulators of GABAergic transmission, both in vivo and in vitro. It was shown that AP is also able to modulate the transcription of genes encoding subunits of the $GABA_A$ receptor (***Follesa et al., 1998; Concas et al., 1998***). In fact, high AP physiological levels that occur, for example during pregnancy, induce variations in the structure and function of the $GABA_A$ receptor in the rat (***Follesa et al., 1998; Concas et al., 1998; Smith et al., 1998; Fenelon and Herbison, 1996***). Changes in brain and plasma levels of AP are influenced by environmental conditions. In particular, some acute stress models such as foot-shock, swim stress or some experimental manipulations, induce in animals a significant increase in brain and plasma concentrations of AP (***Purdy et al., 1991; Barbaccia et al., 1996, 1997***). This effect is similar to that induced by drugs that reduce GABAergic neurotransmission, such as isoniazid, an inhibitor of GABA synthesis (***Horton et al., 1979***) and the anxiogenic β-carbolines (***Barbaccia et al., 1996***). Similar to foot-shock stress, acute administration of ethanol induces an increase in brain and plasma levels of AP (***Rivier et al., 1984; Morrow et al., 2001***). Acute administration of ethanol elicits multiple effects on the central nervous system, similar to those of positive modulators of $GABA_A$ receptor drugs such as benzodiazepines and barbiturates. Ethanol, in fact, has mainly anxiolytic, anticonvulsant and sedative-hypnotic effects. In addition, alcohol causes lack of motor coordination and, at high doses, acts as an anesthetic. It has been hypothesized that the increased levels of AP induced by ethanol may contribute to its central actions associated with the modulation of GABAergic transmission. In fact, the administration of finasteride, an inhibitor of AP synthesis, prevents the increase in cerebrocortical AP levels induced by ethanol, as well as some of its pharmacological effects in rats (***Van Doren et al., 2000***). The response mechanism through which stress and ethanol

increase plasma and brain concentrations of AP is mediated by the HPA axis. In castrated rats without the adrenal glands, foot-shock stress or the administration of ethanol are not able to modify brain concentrations of AP (***Barbaccia et al., 1997; Khisti et al., 2002a, 2002b***). Similarly, (***Chappel et al., 1986; Moldow et al., 1987; Owens and Nemeroff, 1991***), it has been shown that ethanol stimulates the HPA axis by inducing the secretion of CRF (***Ellis, 1966; Rivier et al., 1984; Ogilvie et al., 1997***).

Ethanol

Chemical structure

Ethanol, chemically, belongs to the group of alcohols, organic compounds with different alkyl chains and with a hydroxyl (-OH) as a functional group bound to a non aromatic carbon atom (Fig. 4). Ethanol is an organic molecule consisting of a short aliphatic chain with two carbon atoms and a hydroxyl group. The simultaneous presence of the components, hydroxyl and ethyl, confers to the molecule both hydrophilic and lipophilic properties; ethanol is therefore an amphoteric substance, an important feature of its biological activity. Ethanol is completely soluble in many organic solvents and has a great ability to dissolve in water, due to its short alkyl chain; it is also able to easily cross lipid membranes.
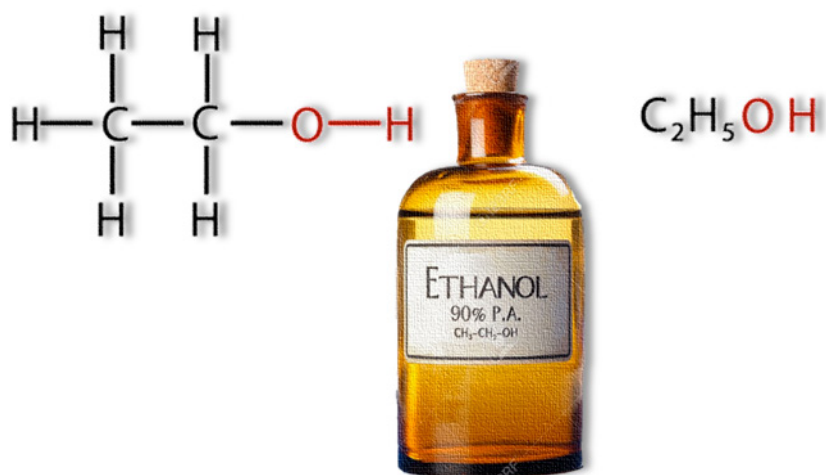

Figure 4 Chemical and molecurar structure of ehanol

Recreational use and pharmacology of ethanol

From a socio-cultural point of view, ethanol, produced in nature by fermentation of sugar, is a substance that has always accompanied human society since ancient times. In fact, there are records of alcoholic beverages dating back to 3000 B.C. Due to its typical disinhibiting effects, infact it is used during important social events or moments of daily routine. Its easy availability and relatively low price led ethyl alcohol to become the most widespread substance of abuse in the world.

Ethanol is taken orally, absorbed in the intestine, and has a distribution volume close to that of total body water. It is metabolized primarily in the liver, first to acetaldehyde by the action of alcohol dehydrogenase and then to acetate by aldehyde dehydrogenase. Excretion occurs mainly through the kidney, but a part is excreted through the lungs (Fig 5)
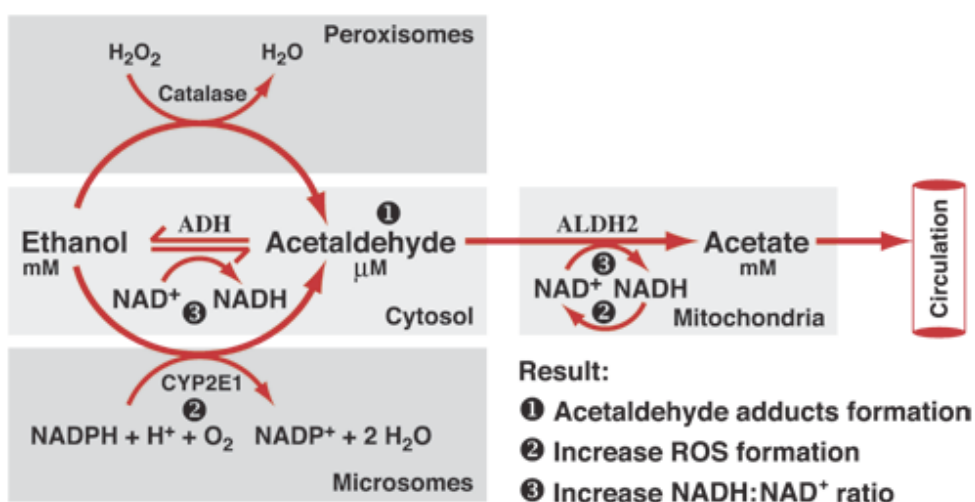

Figure 5 Alcohol metabolism

Neuropharmacologically, ethanol is a psychoactive drug; it is a central nervous system depressant that, in increasing doses, causes sedation and, in the end, hypnosis. The pharmacological activity of ethanol is always inhibitory, although at low concentrations the disinhibiting anxiolytic effects predominate. By increasing the dose, the sedative-hypnotic and anticonvulsant effects may occur. The molecular mechanism of this action at the central level has not yet been completely established.

Like all substances of abuse, ethanol is able to induce tolerance and dependence after chronic intake. Tolerance is defined by a reduction in the pharmacological effects, which leads alcoholics to gradually increase the dose to achieve the same initial effect. Dependence instead is primarily physical, and is determined by the occurrence of withdrawal symptoms following discontinuation of chronic administration. The withdrawal syndrome is a clinical state with several stages that may include tremors, nausea, insomnia, hypertension, anxiety, depression, dysphoria, hallucinations and convulsive disorders. These symptoms in heavy drinkers occur within a few hours after drinking cessation, and persist for up to 24-48 hour after withdrawal. In severe cases, withdrawal symptoms occur in association with other complications such as infections, malnutrition and electrolyte imbalance. In this case, the appearance of delirium tremens, is characterized by severe clinical manifestations and disorganization of behavior can occur. In this situation, the person develops agitation, insomnia, confusion, disorientation, visual hallucinations, designing and generalized seizures disorders. Clinically hyperhidrosis, tachycardia, hypertension, hyperthermia, tachypnea, photophobia and, less frequently, nausea and vomiting occur.

The development and improvement of therapeutic methods available today to treat diseases related to alcohol abuse are closely dependent on an understanding of the mechanisms of ethanol action, and of the knowledge of the biological mechanisms responsible for the

adaptive modifications that develop as a result of chronic ethanol intake. The first hypothesis on the mechanism of action of alcohol was based on the idea that this substance could act through biological membranes thus altering the physical-chemical properties of the cell, due to its ability to dissolve in water and to enter in a lipid-rich environment. This hypothesis, called the *"lipid theory"*, has changed over the years, to make place for new concepts according to which alcohol would have a direct effect on membrane proteins. Nowadays, it is thought that ethanol acts on several neurotransmitter systems, although some of these seem to be more sensitive to its action, such as glutamate, glycine, cholinergic and GABAergic receptors. It is known that ethanol acts on the central nervous system (CNS), altering synaptic neuronal transmission, with consequent reduction in the speed of cerebral activity. Effects on ion channels, both voltage and receptor-dependent, are particularly important. Specific effects that alcohol exert on functionality of these channels are modulated both by subunit composition and phosphorylation status of these ion channels. In recent decades, numerous experimental data have shown that ethanol acts at the neuronal level by altering the function of specific membrane receptors for the neurotransmitters, the activity of which modulates brain functions including complex behaviors. Several studies have also shown that ethanol acts centrally on a number of neurotransmitter systems, for example on the dopaminergic system, noradrenergic, serotoninergic and, in particular, on the GABAergic system, as well as on some hormonal systems.

Among all the neurotransmitter systems involved in the development of alcoholism and other acute or chronic effects, it seems that the excitatory system mediated by glutamate and inhibitory GABAergic transmission play a crucial role in some of the acute and chronic effects induced by ethanol consumption. GABAA receptors in the CNS mediate a number of typical effects of ethanol and, due to their plasticity, show a modification of their subunit

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composition in response to physiological and functional needs of the cell. Therefore, it can be said that ethanol has an inhibitory activity on the CNS in mammals, and that many of its pharmacological actions are similar to those possessed by the benzodiazepines and barbiturates, drugs that enhance GABAergic transmission through activation of GABAA receptors (***Metha and Ticku, 1999***).

Ethanol and neurosteroids

An increasing literature suggests that many of the acute pharmacological actions of ethanol are mediated by an increase in brain levels of neuroactive steroids (***Morrow et al., 1999***) derived from the metabolism of progesterone. It has been reported above that the acute systemic administration of ethanol in the rat is able to induce marked increases in the AP steroid concentrations in the plasma, in the cerebral cortex and hippocampus (***Barbaccia et al., 1999; Van Doren et al., 2000; Morrow et al., 2001***). Furthermore, evidence shows that the treatment of animals with finasteride, an inhibitor of the enzyme 5α-reductase and thus the biosynthesis of allopregnanolone, decreases the increased levels of AP induced by ethanol, and prevents some neurochemical, electrophysiological and behavioral changes, such as the hypnotic effect (***Van Doren et al., 2000; Khisti et al., 2002***). This evidence leads us to believe that ethanol might exercise its cognitive and behavioral effects through an indirect mechanism of increased neurosteroid levels that positively modulate GABAergic transmission. It seems that the major mechanism by which ethanol induces an increase in AP levels is a stimulating effect on HPA axis function (***Khisti et al., 2003; Biggio et al., 2007***). In rats deprived of adrenals, alcohol does not induce to increases in plasma AP nor its typical behavioral effects

(***Khisti et al., 2002, 2003***). Taken together, all these findings suggest that neuroactive steroids synthesized in peripheral organs in response to activation of the HPA axis may be responsible for some effects of alcohol on GABA$_A$ receptors (***Biggio et al., 2007***) (Fig. 6).

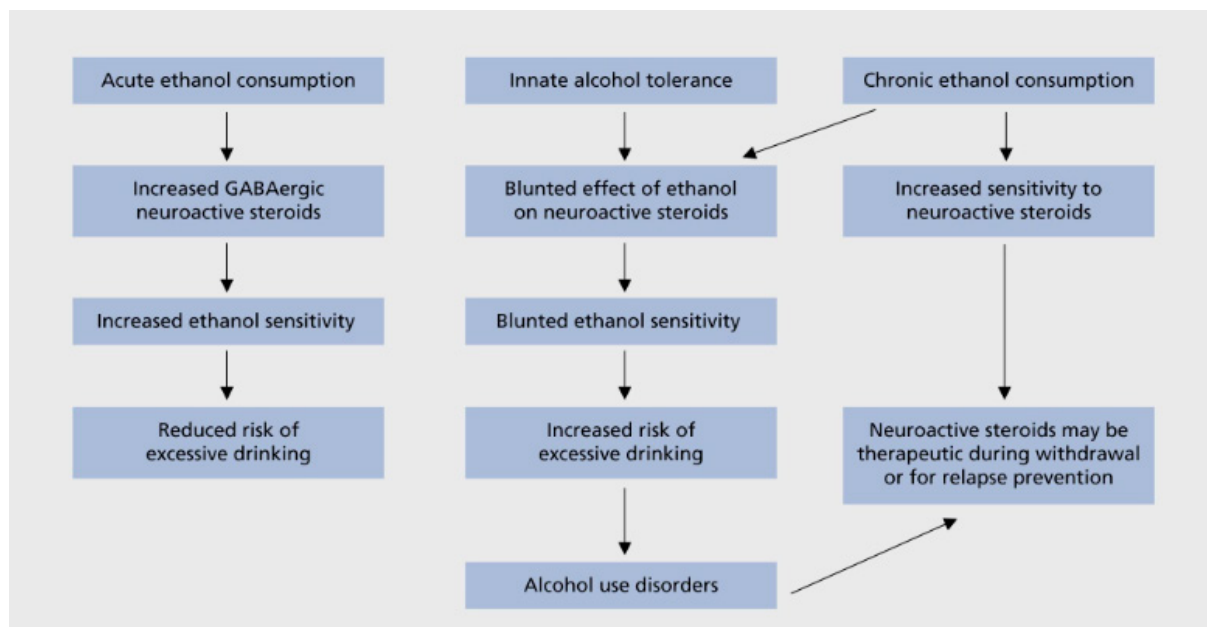*Figure 6. Hypotetical role of neuroactive steroids in alcoholism (Morrow et al., 2006)*

Given that neuroactive steroids can also be synthesized de novo in the brain, independently of peripheral organs, these mechanisms could be induced by alcohol directly in the brain. In hippocampal slices we found direct evidence that neurosteroids contribute to the enhancement of GABAergic synapses induced by ethanol. It appears that this substance has a direct postsynaptic effect on GABA$_A$ receptors, albeit temporary, showing a marked acute tolerance within 10 minutes (***Sanna et al., 2004***). However, a slow development of a postsynaptic enhancement, mediated by the release of neurosteroids, causes the re-emergence of an increase in postsynaptic GABAergic neurotransmission; in addition, ethanol exerts a marked effect on the facilitatory presynaptic release of GABA independent from the action of

neurosteroids (***Sanna et al., 2004***). These findings reveal a complex interaction between ethanol, neurosteroid biosynthesis and regulation of GABAergic transmission.

Ethanol and pregnancy

Alcohol abuse during pregnancy is still an unsolved problem. Alcohol crosses freely the placenta and enters the fetal circulatory system (***Waltman and Iniquez 1972***). Due to the reduced activity of enzymes that metabolize ethanol, the fetus is unable to metabolize it, thus resulting in an increase in its concentration in the amniotic fluid (***Brien et al. 1983***).

FAS (fetal alcohol syndrome) is the main consequence of alcohol abuse during pregnancy; the diagnostic criteria are represented by the presence of well-defined morphological features, such as cranio-facial malformations, delay in pre- and post-natal growth and obvious dysfunction of the central nervous system, including cognitive and behavioral deficits (***Jones and Smith 1973***). FASD is the term used to include the fetal alcohol spectrum disorders that affect children who do not fulfill the criteria for FAS, but have deficits linked to prenatal alcohol exposure (***Riley and McGee 2005***). Several studies conducted both in humans and in animals have shown that prenatal exposure to ethanol can be determined in infants impair motor activity, attention deficit and also disorders during the lactation phase (***Martin et al., 1979***). In particular, infants show an alteration in breast sucking and, in rodents, there is a reduction of vocalizations; since the vocalizations are important in evoking the attention of the mother, reducing vocalizations seems to have important implications for the puppies in terms of nutritional intake and in behavioral development. Vocalizations are indeed important in social behavior such as aggression, game and mother-child interactions, behaviors that are

altered upon prenatal exposure to alcohol (***Brudzynski 2005***). The different susceptibility of children to the effects of prenatal exposure to alcohol is also due to the interaction of alcohol with other risk factors, such as the dose and the exposure time, polydrug use, exposure to environmental toxic agents, stress-related hormones and some genetic variants (***Abel and Hannigan 1995***). Particularly, prenatal stress in humans can cause a variety of disorders similar to those caused by prenatal exposure to alcohol, including low birth weight, developmental delay, anxiety disorders, depression and ADHD (***Beijers et al., 2010; Wadhwa 2005; Van denBergh and Marcoen 2004***). Accordingly, recent animal studies show that prenatal stress may enhance the effects of prenatal exposure to alcohol (***Roberts et al. 2004; Schneider et al, 2002, 2004, 2008, 2011***).

Beyond the serious behavioral, cognitive and structural deficits of FASD children, one of the most commonly described disabilities both in childhood and adulthood is the presence of a high rate of mental disorders, including depression and anxiety disorders (***Barr et al., 2006; Cryan et al., 2002; Famy et al., 1998; Streissguth et al., 1994***). In addition, several studies have shown that prenatal exposure to alcohol might cause hyperactivity and dysregulation of the HPA axis; for example, the intake of high amounts of ethanol during pregnancy has been associated with high basal levels of cortisol (***Jacobson et al., 1999; Ramsay et al., 1996***) and with an increase in the activity of the HPA axis in response to stress (***Haley et al., 2006***) in children during the first months of life. Studies based on animal models support these findings; rats prenatally exposed to ethanol do not usually show changes in the basal levels of corticosterone and adrenocorticotropin, but show, when exposed to acute stress, a hyperactivation of the HPA axis (***Kim et al., 1999; Weinberg, 1988, 1992, 1993; Weinberg et al., 1996***). The HPA axis dysregulation is the biological alteration most consistently observed

in depression and anxiety disorders. This could then explain the emotional disorders observed in these subjects (**Hellemans et al., 2010**).

Zimmerberg and Brown have studied the effects of a postnatal stress, such as separation from the mother during the first week of life, on the response to an acute stress in adult animals who were prenatally exposed to alcohol by measuring the levels of allopregnanolone, showing that the combination of the two stressors increases sensitivity to steroidogenic effects induced by stress compared to animals that have not been separated from their mother.

Another possible consequence of prenatal exposure to alcohol is that there may be an increased risk of ethanol abuse during adolescence and in adulthood. In fact, clinical studies on experimental animal, epidemiological and experimental trials on humans show that prenatal or neonatal alcohol exposure can affect the subsequent abuse of ethanol during adolescence (**Spear and Molina 2005**). In addition, ethanol consumption during pregnancy could modify maternal care (**Pepino et al., 2002**), important in the psychological and physiological development of the child. These effects can also persist in adults. More specifically, a reduction of one of the behaviors that define the affection of mothers, such as the "licking-grooming", results in an early onset of puberty (**Borrow et al., 2013; Cameron et al., 2008**), an increase of stress responsiveness (**Cameron et al., 2005**) and alcohol abuse (**Francis and Kuhar 2008**). Pueta et al. (2008) have shown the effects of exposure to moderate doses of ethanol during pregnancy and higher doses in the postpartum period, on the interaction of mothers with their pups, showing how the administration of ethanol in the postpartum period alters maternal behavior.

Although, it is widely accepted that exposure to high doses of ethanol has lasting negative effects on brain development, the case for moderate exposure remains controversial. Several models of FASD in primates and rodents have convincingly demonstrated the significant

effects of exposure to alcohol in different regions of the brain. These studies showed that exposure to moderate doses may alter the neurotransmission and neuromodulation systems throughout the brain, leading to neurobehavioral changes in offspring (***Valenzuela et al. 2012***). Cullen et al. (2013) evaluated the effects of long-term exposure to a low dose of ethanol during pregnancy on the anxious behavior in adult offspring, showing that alcohol consumption (albeit at relatively low doses) in pregnancy can alter brain development and contribute to an altered behavior in offspring.

It is very difficult to define the minimum dose that has effects on the developing fetus and will cause all the anomalies described; there is no precise dose, so it is extremely important not to take alcohol during pregnancy (***Ornoy and Ergaz , 2010***).
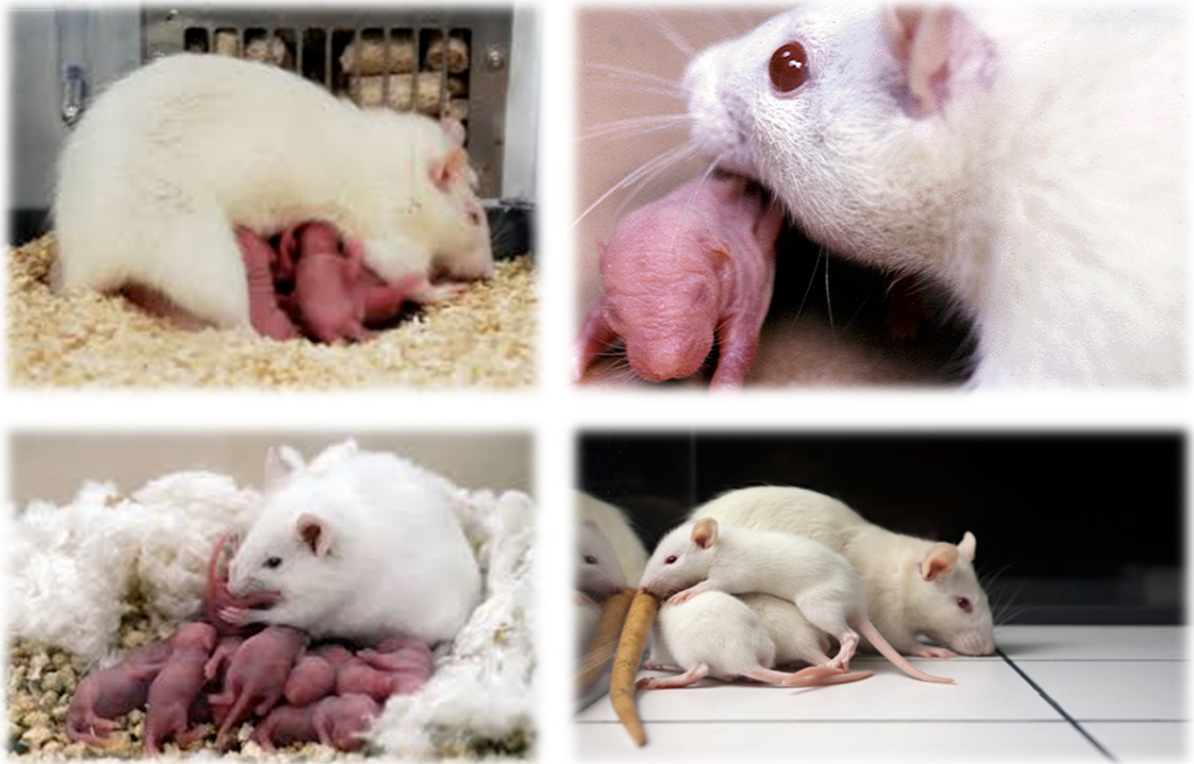
Maternal care

Social interaction and stimuli to which humans, but also other animal species such as rats, mice and monkeys, are exposed at an early age, deeply influence neurobiological and behavioral development. In particular, maternal care plays an important role in development and can affect the psychobiological side of the offspring (***Harris, 1999***).

Transgenerational transmission of maternal care has been demonstrated in rodents (***Champagne, 2008***). They show well-defined maternal behaviors, such as licking-grooming (the pups are licked in the anogenital and peri-oral area) and arched-back nursing (lactation standing with her back arched); these behaviors, according to the scientific literature, reflect the "affectionate" behavior of mothers (***Myers et al. 1989; Levine, 1994***), which regulates the physiological functions of children and has effects on the development of the central nervous system (***Liu et al., 1997***). In rodents, maternal care begins at birth, under the pressure of the changing endocrine conditions that occur during delivery. That is, progesterone levels abruptly drop and levels of prolactin and estrogen increase. Nevertheless, hormonal changes alone are not sufficient to ensure the maintenance of maternal care. Hormonal changes in the last phase of pregnancy and during the post-partum period activate the neural circuitry of maternal behavior that involves different brain areas (the medial preoptic area of the hypothalamus, the lateral septum, the core of the nucleus accumbens and amygdala). Several data in literature (***Champagne, 2008; Pereira et al., 2009***) showed that this circuit is deeply involved in maternal care and consequently in the behavior of pups related to smells, sounds and tactile sensations. Moreover, also the dopaminergic system (thus the reward system) is involved, inducing in a mother a really strong motivation to continue the maternal care, even when hormonal levels decrease (***Clinton et al., 2007***). During gestation, in fact, the interaction between mother and fetus is critical for growth and development and changes during this

period could alter the physiological and psychological integrity of future births. The pre- and post-natal period is an opportunity for the mother to influence, through mother-child interactions, certain characteristics of their offspring by different mechanisms. The maternal care received during the postnatal period induce deep changes in the development of the neural system that regulates the response to new stimuli and to social behaviors (*Meaney, 2001*). Early stress, such as reduced maternal care or maternal separation, are the cause of increased sensitivity of the noradrenergic and serotonergic system, that persist throughout life (*Francis et al., 1999*), such as increased norepinephrine turnover in medial prefrontal cortex (*Pei, Zatterström and Fillenz, 1990*) and the reduction of the levels of the 5HT1B receptor in the hippocampus (*Kikusui et al., 2005*). Given the importance of noradrenergic and serotonergic systems in the etiology of psychiatric disorders such as anxiety, depression, schizophrenia and bipolar disorder, these findings suggest that an alteration of these mechanisms during early life events can make an individual more vulnerable to psychiatric disorders (*Heim et al., 1997*). Liu et al. (1997) suggest that the effects of the first environment on the development of the stress response mediated by the HPA axis reflect a natural plasticity that is well manifested in the postnatal period with maternal care, which is able to activate the first biological response to adverse stimuli. This plasticity would allow the animal to adopt defensive systems to the specific requirements of the environment, through a development of CNS responses to stress in the early stages of life (*Shear, Brunelli and Hofer, 1983, Lichtman and Cramer, 1989*). Maternal behavior therefore affects behavioral development and endocrine responses to stress in the offspring and thus changes in maternal care are the basis of a non-genomic transmission of individual differences in the behavioral response to stress (*Liu et al., 1997; Francis et al., 1999*).

Figure 7 Maternal care in rats

Maternal separation

As previously mentioned, maternal separation is an early stress to which the offspring can be subjected in the postnatal phase (***Francis et al., 1999***). There are several experimental protocols of maternal separation in rodents, which have different variables such as the time of separation or separation mode (for example, remove the mother and leave the litter in the nest, or remove the litter and leave the mother alone in the nest, or even both the mother and the litter are left in the same cage and separated by a transparent plastic panel so as to remain in a visual and auditory contact but without the possibility of any physical contact). Among them, the Early Maternal Separation is a procedure that involves the separation of pups from the mother for 15 minutes a day for a few days. Compared to control rats, which have not undergone any handling, the EH animals possess a minor axis sensitivity HPA, (they are less reactive, more resilient) if subjected to an acute stress (such as foot-shock), both during the childhood and adulthood (***Meaney et al., 1993***).

Furthermore, The EH mothers when they are reunited with separated pups, show an increase of behaviors "affectionate" like licking-groming and arcked-back nursing (***Smotherman et al., 1977***). This increase is related to a reduced activity of the HPA axis; This would bring the pups to be less anxious. These data therefore demonstrate that the Early Maternal Separation renders the animal more adaptable and more resilient to stress.

In our lab, we used the Prolonged Maternal Separation (MS) paradigm. Compared to the Early Maternal Separation, MS varies the duration of the separation; in fact, in this procedure the litter is separated from the dams for 3 hours per day. Whereas the effects of Early Maternal Separation are well documented, replicated and accepted by the scientific community, the effects of Prolonged Maternal Separation are controversial and often conflicting with each other. This is due to the different separation procedures used in some

studies (***Lehmann and Feldon, 2000***), the differences in the frequency, duration and age of animals at the beginning of the procedure, or more in different strains of rats studied that can not be compared with each other, making therefore almost impossible to reconcile the differences that appear in the literature. However, the predominance of data suggests that adult rats subjected to MS at an early age have HPA axis hyperactivity and an increase in CRH levels in the paraventricular nucleus of the hypothalamus and in the median eminence, all indexes of an anxious state, compared to EH. The MS animals show a reduction in exploration if exposed to a new environment, a reduction of the time spent in the open arms in the Elevated Plus Maze test, an increase of the freezing behavior in response to an acoustic stimulus in the Open Field and an increase of the immobility in the Forced Swim Test; these are all indexes of a non-social behavior, fear, anxiety, despair and depression (***Aisa et al., 2007; Ladd et al., 2000; Huot et al., 2001; McQueen et al., 2003; Veenema et al., 2006; Wigger and Neumann, 1999***). Furthermore, these animals show, in addition to this anxious behavior, an increase in aggressiveness both in adolescence and in adulthood, toward conspecifics; it would therefore appear that MS promotes the expression of aggressive behavior in male rats (***Meaney and Stewart, 1979; Panksepp et al., 1984; Pellis et al., 1987; Vanderschuren et al., 1997; Veenema et al., 2006; 2009***). The effects of MS at an early age have an impact on drug abuse: in fact, studies in mice deprived of contact with the mother show greater vulnerability to cocaine addiction (***Kikusui et al., 2005***). Other studies show that rats deprived of maternal care for a long period of time show a reduction of neurogenesis in the hippocampus, and reduced levels of Brain-derived neurotrophic factor (BDNF), a neurotrophin that promotes neuronal survival and proliferation (***Liu et al., 2000; Mirescu et al., 2004***).

In our laboratories, it has been demonstrated that separating pups from the dams during the first two weeks of life does not alter, in the adult offspring, the functionality and reactivity of the HPA axis.

Instead, it determines a protection against a subsequent long-term stressful experience in the rat. This is demonstrated by the effect of two different stressful stimuli, such as maternal separation and social isolation as a chronic stress, on plasma levels of corticosterone in adult animals; social isolation leads to a drastic reduction in corticosterone levels compared with controls, but this reduction is less pronounced in the isolated animals tested with maternal separation (***Biggio F. et al., 2014***). This result is even more evident when the animals are subjected to an acute stress, such as the foot-shock. In fact, the increase in corticosterone levels in isolated animal is reduced in isolated rats who were also exposed to maternal separation (***Biggio F. et al., 2014***).

Following the same experimental conditions, the levels of allopregnanolone were evaluated. Social isolation decreases, as expected, the levels of plasma AP; this reduction is less pronounced in isolated animals subjected to maternal separation; consistently with the findings on the levels of CTS, the increase in the levels of AP induced by foot-shock in animals socially isolated and subjected to maternal separation is significantly reduced compared to isolated control rats. This suggests that the condition of experienced stress in early childhood might play a protective role and allows adaptation to chronic stress experienced in another critical stage of development (***Biggio F. et al., 2014***).

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Vulnerability to alcohol abuse

As described in preview paragraphs, alcoholism is a chronic disorder characterized by behavioral, physical and psychological changes caused by continuous and uncontrolled consumption of large quantities of alcohol. It is characterized by an obsessive and compulsive research of alcohol. In fact, in order to achieve the same effects, an alcoholic necessitates to increase the doses of ethanol consumed. Thus, alcohol dependent people do not have control on the number and the amount of drinks, because they have a continuous craving toward ethanol consumption.

It is wide accepted that a dysfunction in the reward system can produce a discomfort and, at the same time, an attitude to seek stimuli and therefore the positive reinforcement given by drugs such as ethanol. Furthermore, the familiar and socio-cultural environment could become a really important factor that could bring vulnerable people to an alcohol dependence problem.

There are several factors that may contribute to the development of alcohol dependence. Among them, may consider the following:

✓ Age, sex: men have a higher risk to become addicted than women, even if women are most likely to suffer from diseases linked to drinking, such as liver diseases. Furthermore, drinking since young age leads to increased vulnerability to alcohol abuse, as well as a regular and prolonged ethanol consumption leads to alcohol physical addiction.

✓ Stress and social and cultural factors: living in an environment in which alcohol is strongly used may increase the risk of alcoholism. Furthermore, stressful events could lead to alcohol

abuse. It is well known that drinking behavior is common in people subjected to stress disorders, such as anxiety disorder and depression.

✓ Genetics: genetic basis determine individual susceptibility to alcohol abuse. The risk of alcoholism is higher in individual who had alcoholic parents.

The improper functioning of the reward system is attributable to environmental and genetic factors (***Dwight and Stoudemire., 1997***). Consumption behavior is then correlated not only to a single neuroendocrine alteration (single mutation), but to a plot of chromosomal polymorphisms capable of acting as contributory factors in determining the level of vulnerability. Hence, susceptibility to alcohol consumption is multifactorial, requiring the involvement of multiple genes, on one hand, and interaction with environmental factors on the other.

Based on these evidences, genes involved in vulnerability to alcohol abuse have been studied. Several genes mutating a copy number variation (CNV) have been identified They could play an important role in addiction.

Furthermore, it was found that these genetic variants are more prevalent in some ethnic groups than in others. Alcoholism is a complex disease that involves different genes whose interactions can lead a vulnerability to the development of alcohol addiction. Variants of each of these genes increase the vulnerability to alcohol, but many of them are widespread differently in the population and generally can affect the way of drinking, other forms of addiction, problem behaviors and stress-related disorders.

Stress, as widely believed, is another factor of vulnerability to alcohol abuse. It is a physiological response to every stressful stimulus of the environment, and consists in a neuroendocrine activation of the CNS and the immune system. This adaptive response is helpful but expensive because it reduces the functional reserve, but, at the same time, could be

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harmful when it is very intense, frequent and / or prolonged, with long-lasting or permanent adverse effects that may result in disorder of adaptation.

Alcohol attenuates the vegetative responses to stressful events, and in virtue of its euphoric effect, it is often used to escape from everyday stress up to the acquisition of habits that can have a detrimental impact on the mental and physical personal maturation.

As clearly discuss, an increasing amount of data suggest that alcohol can lead to an increase of brain levels of neurosteroids, due to the stimulation of the HPA axis. (***Khisti et al., 2003; Biggio et al., 2007)***.

All the stressors that may occur during pre and postnatal period (maternal deprivation, social stress, neglect, physical and sexual abuse of mother and offspring) have been associated to a development of many behavioral disorders in humans and in animals. These disorders include depression and anxiety, and also increase vulnerability to drug abuse (***Moffett et al., 2007***).

A pivotal role for ethanol addiction may be the crosstalk between environment and epigenetic modifications. Epigenetic changes of DNA consist in enrichment of cysteine methylation. Research has shown that the combination of pre and postnatal exposure to ethanol leads to important changes, in the activity the DNA methyltransferase, an enzyme that catalyzes the transfer of a methyl group from S- adenosyl methionine (SAM) to a CpG site of the DNA promoter regions. This process alters the binding of transcriptions factors, reducing the expression of target genes. One gene whose expression is epigenetically regulated is the methyl CpG binding protein (MeCP2). This protein is essential for the normal function of neurons, and seems to be particularly important for their maturation, when it is present in high level and function as a silencer of the DNA in brain (***Perkins et al., 2013***). In summary development of the nervous system is a highly complex process, in which epigenetics plays an crucial role (***Singh et al., 2009)***. Epigenetic regulation of imprinting control regions has

recently become an area of great interest. Imprinted genes regulate both fetal development and expression of these genes is modulated by DNA methylation *(Haycock, 2009)*. Methylation occurs on either paternal or maternal allele, leaving only one allele to be expressed. There are regions that are differentially methylated throughout development, and others that show tissue-specific differential methylation patterns *(Haycock & Ramsay, 2009)*. Cell differentiation (e.g. hippocampal pyramidal vs. granule cell) occurs as a result of tissue-specific methylation patterns. Importantly, methylation of these imprinted control regions is affected by pre-conception (*Knezovich & Ramsay, 2012*) and pre-implantation *(Haycock and Ramsay, 2009*) alcohol exposure. Epigenetic regulation of gene expression has been shown to play a role in developmental processes, and recent research demonstrates epigenetic modifications in the etiology of FASD, using animal models. Importantly, very few studies have focused on alcohol-induced epigenetic changes in the brain, let alone specific brain regions. Moreover, research into long-term epigenetic changes caused by developmental alcohol exposure is very much needed.

Therefore, based on the above, both environment and epigenetic modification plays a fundamental role in the vulnerability to alcohol abuse.

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Aims

   As reported in the introduction section, environmental insults (stress, toxins, infections) during pregnancy as well as the consumption of alcohol during childhood may affect the behavior (***Popoola et al., 2015***). Based on these evidences, the major aims of my work were to

1) Investigate whether ethanol consumption during pregnancy combined with newborn exposure to stress, induced by daily maternal separation, alters a) emotional behavior (anxiety), b) sensitivity to acute stress in adult offspring and c) vulnerability to ethanol consumption.

2) Study the effect of ethanol administration during pregnancy on maternal care behavior and hormone blood levels measuring AP and CTS, as well as elevated plus maze test response in the offspring.

3) Evaluate the responsiveness to an acute stress such as foot shock in offspring measuring AP and CTS plasma levels.

4) Study the role of maternal care and blood CTS levels in dams, in order to understand weather treatment and maternal separation could have any influence in this important parameter.

5) Set up an Ascending Ethanol Concentrations Paradigm, that provides exposure to an increasing concentration of EtOH solution for each daily session, to better understand if the vulnerability to alcohol consumption during adolescence or adulthood will be changed.

Altogether, these results may help to elucidate the mechanisms underlying behavioral changes in animals subjected to a stress during early life or whose mothers underwent to wrong habits during pregnancy such as alcohol consumption. Understanding the mechanisms underlying

these changes may help to find potential new pharmacological strategies to counteract states

of alcohol abuse in adult individuals.

Materials and Methods

Animals

The study was performed using Sprague-Dawley male and female rats (Charles River, Calco, Italy). All animals were housed under an artificial 12 h light (9:15 a.m-9:15 p.m.) – 12 h dark cycle (9:15 p.m.-9:15 a.m.) at a constant temperature of 23 ± 2 °C and 65% humidity. Food and water were available ad libitum. Mating occurred in individual cages with one male of 150 days of age and one female of 120 days of age per cage. Gestational day (GD) 1 was assigned when the copulation plug was detected in the bottom of the mating cage. From GD 12, rats were manipulated once a day for four days, until GD 16 for gavage habituation before the beginning of the protocol. Handling consists of introducing an intragastric cannula connected to a 2.5 cc syringe (containing a vehicle solution) into the stomach through the oral cavity, in order to get animals used to the treatment. Thus, from GD17 up to GD20, a group of dams (EtOH) was treated intragastrically (gavage) with a 1g/Kg ethanol solution, diluted in low fat milk; the control group of dams (VEH) was treated with a solution of low fat milk and water.
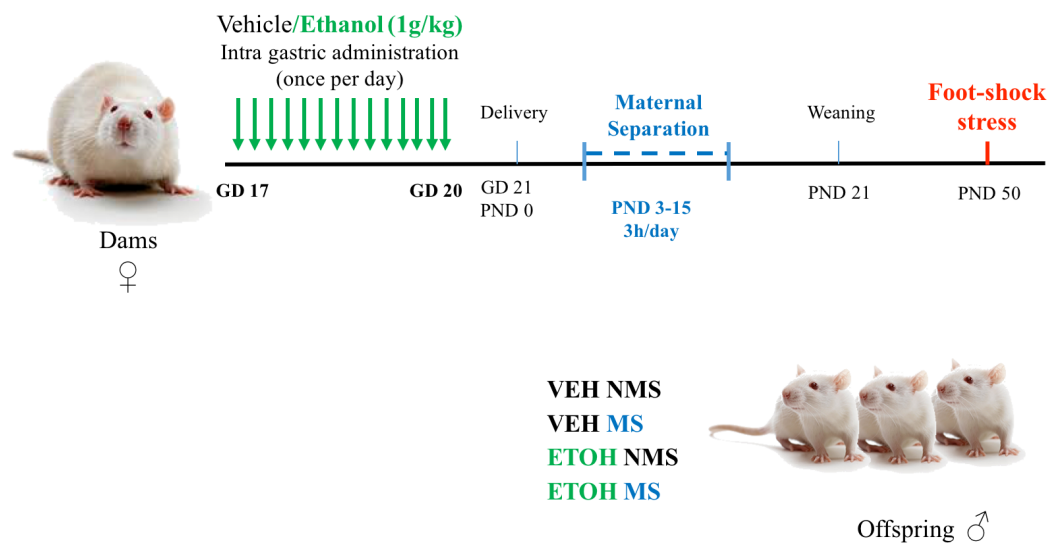
Figure 8: Schematic diagram of the experimental procedure

On GD20, each dam was individually housed in a single cage (40 cm x 60 cm x 20 cm) until delivery. Starting from postnatal day 2 (PND 2), all litters were equally distributed between male and female (5-6 for each sex). Pups used in our experiment were randomly assigned to four experimental groups: offspring from dams treated with vehicle solution not subjected to maternal separation (VEH NMS), offspring from dams treated with vehicle solution subjected to maternal separation (VEH MS), offspring from dams 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). (Figure 8)

Animal care and handling during the experimental procedures were carried out in accordance with the European Parliament and the Council Directive of 22 September 2010 (2010/63/UE) and were approved by the local ethics committee. Moreover, adequate measures were taken to minimize discomfort.

Maternal Separation

According to a previous report (***Plotsky and Meaney, 1993***), the separation procedure consists of daily separation of the litter from dams for 3 hs (10:30 a.m.–1:30 p.m.) from PND 3 until 15. During these 3 hours all litters were transferred to a separate room, to prevent vocal communication with dams that were left undisturbed in their home cage until pup reunion. Pups were placed in cages with soft cotton, and the temperature was maintained at 32–33 °C, consistent with normal nest temperature. NMS pups were handled twice a day and left in the home cage with the mother. After PND 15, pups were returned to normal housing conditions until weaning (23-24 PND), when males were randomly housed in groups of five per cage and maintained under standard laboratory conditions until PND 50, when the experiments started.

Evaluation of maternal care

The behavior of each dam, in terms of maternal care, was observed for four daily 75-min observation sessions, from PND 3 until 15. Observations occurred at the same time each day with a session during the dark phase (8.00 a.m.) and three sessions during the light phase (9.15 a.m., 1.30 p.m. and 4.30 p.m.), with the first two observations before maternal separation (beginning at 10.30 a.m.) and the last two observations after maternal separation (ending at 1.30 p.m.).

The distribution of the observations was based on findings that nursing in rats occurs more frequently during the light phase. For each observation session, behavior of each mother was observed for a brief period (approximately for 5s) 25 times, once every 3 min. Thus, every

dam was observed 100 times per day (25 observations per sessions x four sessions per day = 100 observation/mother/day).

As previously detailed by others (***Myers et al. 1989***), the following behaviors were scored: (1) licking and grooming (LG) any pup, (2) nursing pups in a passive posture or (3) in an arched-back nursing (ABN) posture, (4) building the nest, (5) self grooming, (6) staying in the nest in contact with the pups (in nest), (7) resting with no contact with the pups (resting) and (8) eating or drinking. To provide more reliable estimates of individual differences in maternal behavior, we observed the cohort of 17-20 mothers/litters for each experimental group. Data were analyzed as the percentage of observations in which animals engaged in the target behavior.

Foot-shock stress
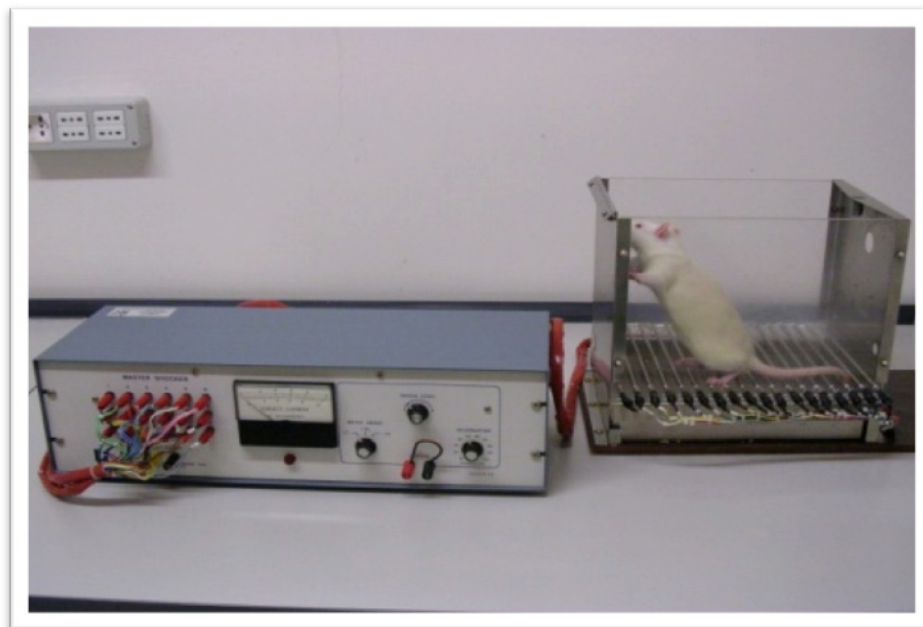
Figure 9: The foot-shock stress apparatus

Animals were exposed to foot-shock stress at PND 50 for steroids assay. Rats were randomly assigned to eight experimental groups: VEH NMS, VEH NMS/stress, VEH MS, VEH MS/stress, EtOH NMS, EtOH NMS/stress, EtOH MS and EtOH MS/stress. The foot-shock stress consists of a series of electrical stimuli delivered in individual boxes with floor made of brass rods positioned 2 cm apart. Shocks (0.2 mA for 500ms) were delivered every seconds over a period of 5 min (Figure 9). Animals were killed 25 min after the end of the foot-shock exposure for allopregnanolone and corticosterone measurements.

Elevated Plus Maze
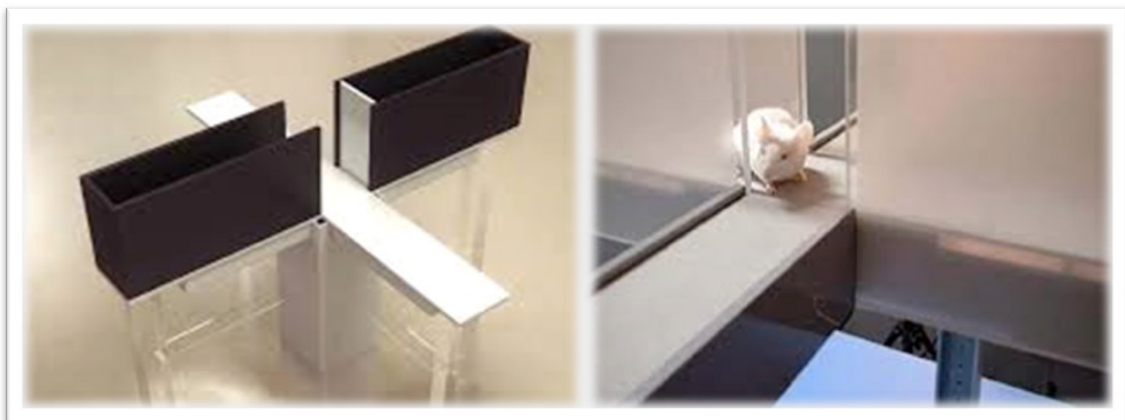
Fig. 10 Elevated plus Maze apparatus

Elevated plus maze was used to test anxiety-like behavior in animals. The plus-maze is a black polyvinyl chloride apparatus, elevated 50 cm above the floor and containing two open arms and two closed arms (12 x 60 x 3 cm), connected by a central square (12 x 12 x 3 cm) (Figure 10). The apparatus is located in a quiet and dimly lit room. Each rat was tested only once, placed on the central square facing towards one of two closed arms and allowed to

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freely explore the maze for 5 min. The numbers of entries into and time spent in the open arms were recorded, with army entry being defined as the presence of all four feet of the animal in the arm.

Measurement of hormone levels

Animals were sacrificed between 10:00 a.m. and 12:00 p.m. with a guillotine, immediately after weaning (dams) and at PND 50 (offspring). Blood was collected from the trunk of sacrificed rats into K3-EDTA tubes, centrifuged at 900 x g for 10 minutes at 4°C and frozen at - 80°C until use. Levels of allopregnanolone and corticosterone were assayed in the same rats.

Allopregnanolone was extracted from plasma as previously described (**Serra et al., 2000**). The combined organic phases were dried under vacuum. The recovery (70-80%) of allopregnanolone through the extraction procedure was monitored by addition of a trace amount (6000 to 8000 cmp; 20-80 Ci/mmol) of [^{3}H] allopregnanolone (Perkin Elmer Italia, Monza) to the plasma samples. Allopregnanolone levels were quantified by radioimmunoassay with a specific antibody generated in sheep as previously described (**Purdy et al., 1990; Serra et al., 2000**).

The enzyme immunoassay was used to quantify plasma levels of corticosterone. ELISA was performed according to the manufacturer's instruction (Corticosterone ELISA, IBL International, Germany) using a 96-well plate that was precoated with a polyclonal antibody against an antigenic site on the corticosterone molecule. The kit also provided a seven-point standard curve using two-fold serial dilutions. Each sample was run in duplicate.

Ascending Voluntary Ethanol Consumption paradigm

According to previous work (***Martinetti et al., 2006***), experimental procedures were conducted in single cages (42.5 x 26.6 x 15.5 cm) in which the animals were transferred for the 1h daily drinking session consisting in the free choice to consume tap water or EtOH from two different bottles. Before starting the experiment, rats were given free access to water.

After a 2-week habituation period in a reverse dark/light cycle (dark at 10.00 a.m. and light at 22.00 p.m.) the EtOH-access protocol begins and was conducted every day for approximately 55 days. All EtOH solutions (v/v) at each concentration (0.01% to 20%) were prepared with 99.8% (v/v) EtOH (Fluka. Sigma-Aldrich) and tap water. Bottles containing EtOH solution and tap water were weighed on an electronic scale (Kern).

On day one of EtOH access, rats were removed from their home cages and placed in smaller cages, one per cage, in order to receive the two bottles, one containing tap water and the other containing a 0,01% ethanol solution. The concentration of the EtOH solution follows an increasing trend, throughout the treatment period (55 days), everyday until the concentration of 20% was reached in the last session according to the following schedule: 0,01%, 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***). Drinking session began at 10 a.m. every day. Bottles position was inverted every day to avoid position preference, and the weight of each bottle was recorded before and after each drinking session. Protocol was set up in adolescent and adult animals starting at 30 days and 90 days of age respectively.

The last day of the drinking session animals were sacrificed during the dark period of the inverted cycle, when they still had access to the 20% ethanol solution. Blood was collected

from the trunk of sacrificed rats into K3-EDTA tubes, centrifuged at 900 x g for 10 minutes at 4°C and frozen at - 80°C until use.

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.). Differences between groups during the ethanol consumption protocols were assessed with a repeated measure analysis of variance (ANOVA), followed by a Newman-Keuls post-hoc test (Statistica 6.0, StatSoft Inc.). A p<0.05 value was considered statistically significant.

Results

1. Effects of prenatal ethanol exposure and maternal separation on plasma levels of corticosterone and allopregnanolone in adult male rats

In the first step it has been evaluated the effect of two different stressors, prenatal ethanol exposure and maternal separation, when applied separately or given together, by measuring blood levels of AP and CTS in adult male rats. Figure 11A shows that only the combination of the two different stressors leads to a significant decrease in AP plasma levels (*p<0.05* vs V*EH NMS)*. ANOVA revealed a significant effect of prenatal ethanol treatment [F(1, 28)= 10.0756, p= 0,003634] and maternal separation [F(1, 28)= 4.7862, p=0.037197 and no significant interaction between factors [F(1, 28)= 2.5098, p= 0.124369]. Comparison between groups show that the association of different stressors causes a decrease of AP levels compared with VEH MS suggesting that MS alone is not effective on modulating the basal levels of AP in adult animals.

Data related to blood levels of CTS are more consistent since both MS and prenatal treatment with ethanol decrease the levels of CTS significantly when compared with VEH control group (Fig 11B). Similarly to what observed for AP levels, the association of two different stressors did affect the levels of CTS compared to VEH NMS group but not compared with EtOH NMS group, where those levels are already decreased (Fig. 11B). ANOVA comparison between groups showed a significant effect of ethanol prenatal treatment [F(1, 29)= 4.8394, p= 0,035936] and maternal separation [F(1, 29)= 9.06640, p=0.005350, but no significant interaction between factors [F(1, 29)= 0.95501, p=0. 336529].
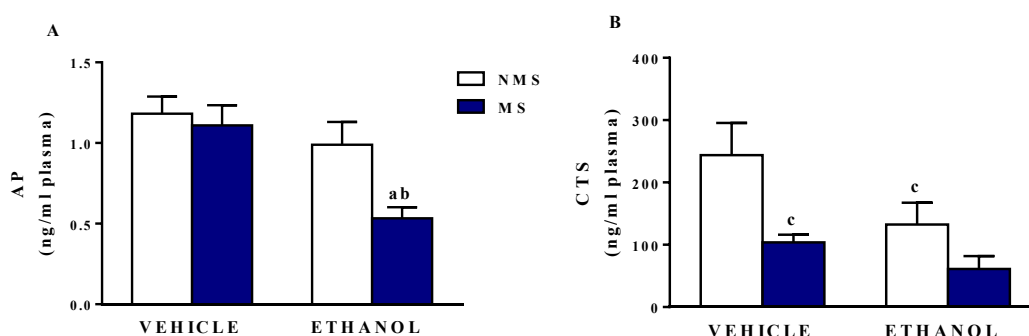
*Figure 11: Two months-old rats were divided into 4 experimental groups: non prenatally ethanol exposed not subjected to maternal separation (VEH NMS), non prenatally ethanol exposed subjected to maternal separation (VEH MS), prenatally ethanol exposed not subjected to maternal separation (EtOH NMS) and prenatally ethanol exposed subjected to maternal separation (EtOH MS). **A**. Data are means ± SEM of values from eight to ten rats $^{a}p<0.05$ vs VEH MS $^{b}p<0.05$ vs ETOH NMS. **B**. Data are means ± SEM of values from eight to ten rats $^{c}p<0.01$ vs VEH NMS. All data were analyzed with a two-way ANOVA followed by Newman–Keuls post-hoc test*

2. Effects of prenatal ethanol exposure and maternal separation on AP and CTS levels induced by acute stress in adult male rats

As previously demonstrated, animals subjected to MS and social isolation stress together are less responsive to acute stress, such as the foot-shock (***Biggio F. et al., 2014***) suggesting that MS may affect the responses to acute stress in a protective manner. Based on these evidences, we wanted to evaluate here the potential protective effect of MS in rats prenatally exposed to ethanol during the last week of pregnancy on acute stress responsiveness. In order to evaluate the stress response, we measured AP and CTS plasma levels 30 min after exposing animals to foot-shock stress.

As expected (Figure 12A), foot-shock stress increased the percentage of AP blood levels in VEH-NMS, VEH-MS and EtOH-NMS groups in a similar manner compared to animals not shocked. When animal subjected to both stress were tested, this increase is much more

evident compared to VEH-MS and EtOH-NMS *(p<0.001)*. ANOVA revealed a significant effect of ethanol prenatal treatment [$F_{(1, 27)}$= 22.4608, p=0,000061], maternal separation [$F_{(1, 27)}$= 6.9229, p=0,013891], and interaction between factors [$F_{(1, 27)}$=18.5052, p=0,000198].

Foot-shock caused an increase of CTS levels in all experimental groups compared to not shocked counterparts (Figure 12B). The enhanced levels of plasma CTS induced by the acute stress is more consistent in animal exposed both to prenatal treatment with ethanol and to subsequent maternal separation compared to that observed in animals not subjected to both stressors *(p<0.001)*. ANOVA revealed a significant effect of ethanol prenatal treatment [$F_{(1, 32)}$=41,1047, p=0,000000] and maternal separation [$F_{(1, 32)}$=98,0813, p=0,000000]. There is no significant effect between factors [$F_{(1, 32)}$=1,6435, p=0,209054].

Interestingly, the increase in the percentage of CTS in VEH-MS group (t-Test, p< 0.05 vs EtOH group) (335.4 ± 36.7 % vs VEH-NMS), is more pronounced than the increase of plasma CTS levels in EtOH-MS group (224.5 ± 24.29 % vs VEH-NMS
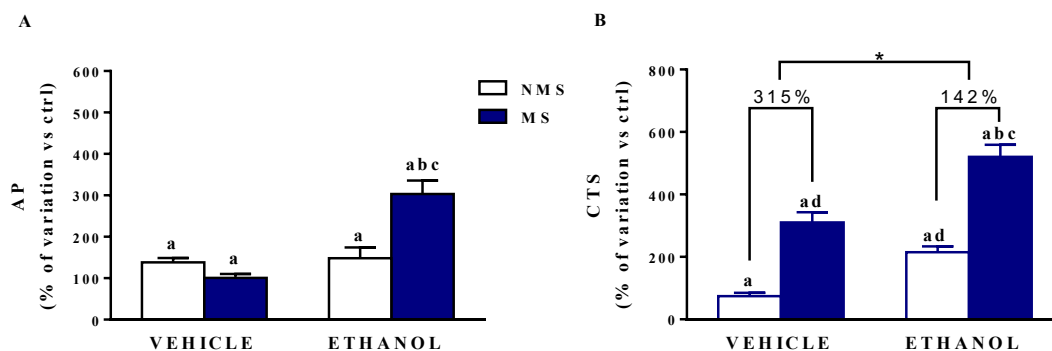
Figure 12: Two months-old rats were divided into 4 experimental groups: non prenatally ethanol exposed not subjected to maternal separation subjected to foot-shock (VEH NMS FS), non prenatally ethanol exposed subjected to maternal separation subjected to foot-shock (VEH MS FS), prenatally ethanol exposed not subjected to maternal separation subjected to foot-shock (EtOH NMS FS) and prenatally ethanol exposed subjected to maternal separation subjected to foot-shock (EtOH MS FS). A. Data are means ± SEM of values from eight to ten rats [a]p<0.001 vs respective NFS, [b]p<0.001 vs VEH-MS, [c]p<0.001 vs EtOH-NMS. B. Data are means ± SEM of values from eight to ten rats [a]p<0.001 vs respective NFS, [b]p<0.001 vs VEH-MS, [c]p<0.001 vs EtOH-NMS, [d]p<0.001 vs VEH-NMS. [*]p<0.01 vs foot-shock-increased % of VEH group (T-test).

3. Effects of prenatal ethanol exposure and maternal separation on anxiety state in adult male rats

It is widely accepted that changes in steroids levels induced by stress are related to parallel modifications in anxiety state. In order to evaluate whether prenatal EtOH exposure, MS or their association impair anxiety in our different experimental groups, we exposed the animals to the elevated plus maze test. Parallel to what observed for AP but not for CTS, elevated plus maze revealed that the association of prenatal exposure to ethanol and subsequent early maternal separation is capable of inducing changes in the emotional state of EtOH-MS group, compared to the counterpart EtOH-NMS. As shown in figure 13, the association of these two stressors induced a marked decrease of preference for the open arms in EtOH-MS animals (p<0.05). ANOVA revealed a significant effect of maternal separation [F(1, 66)= 5.8061, p=0.018767] and no significant effect of ethanol prenatal treatment and between ethanol

prenatal treatment and maternal separation. Furthermore, these animals showed no significant

differences in the number of total entries in the arms.
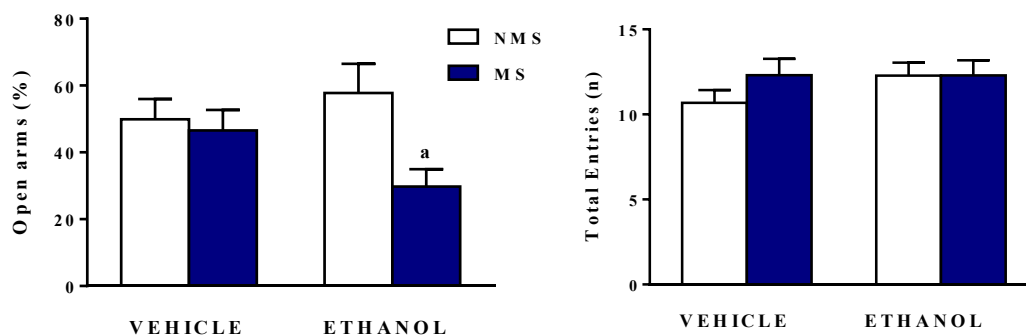

Figure 13: Two months-old rats were divided into 4 experimental groups: non prenatally ethanol exposed not subjected to maternal separation (VEH NMS), non prenatally ethanol exposed subjected to maternal separation (VEH MS), prenatally ethanol exposed not subjected to maternal separation (EtOH NMS) and prenatally ethanol exposed subjected to maternal separation (EtOH MS) Data are means ± SEM of values from fourteen to twenty rats. [a]p<0.05 vs EtOH-NMS. All data were analyzed with a two-way ANOVA followed by Newman–Keuls post-hoc test.

4. Effect of ethanol treatment and pup separation on maternal care

Several studies show that the quality of maternal care has a crucial role in the development of

cerebral function and on HPA axis stress responsiveness in the offspring (***Chamapagne et al., 2003; 2008***). In this study, we examined four daily sampling sessions (75-min duration) of

maternal care parameter consisting in arched back nursing (ABN) and pup licking and

grooming (LG) frequencies in all dam across the first two weeks of postpartum.

ABN and LG are important parameters to discriminate the quality of maternal care behavior

(***Caldji et al., 1998; Champagne et al., 2008***). As shown in figure 14, pup-separated dams in

VEH MS and EtOH MS groups displayed an increase in maternal ABN and LG behavior than

non pup separated dams in VEH NMS and EtOH NMS groups during all the observation

period. Furthermore, as expected, pup-separated dam groups showed a significant increase in

active nursing (LG) compared to non pup-separated dams in the hours following the reunion after maternal separation. ANOVA shows a significant effect of maternal separation on LG frequency [$F_{(1, 480)}$= 39.444, p=0.000000] and of ethanol prenatal treatment [$F_{(1, 480)}$= 7.829, p=0.005347], but no significant effect between factors [$F_{(1, 480)}$=0.155, p=0.693927]. For the ABN behavior, ANOVA revealed a significant effect of maternal separation [$F_{(1, 480)}$= 38,8396, p=0,000000] and no significant effect of ethanol prenatal treatment [$F_{(1, 480)}$= 2,0431, p=0,153546] and between factors [$F_{(1, 480)}$= 0,9164, p=0.338893].
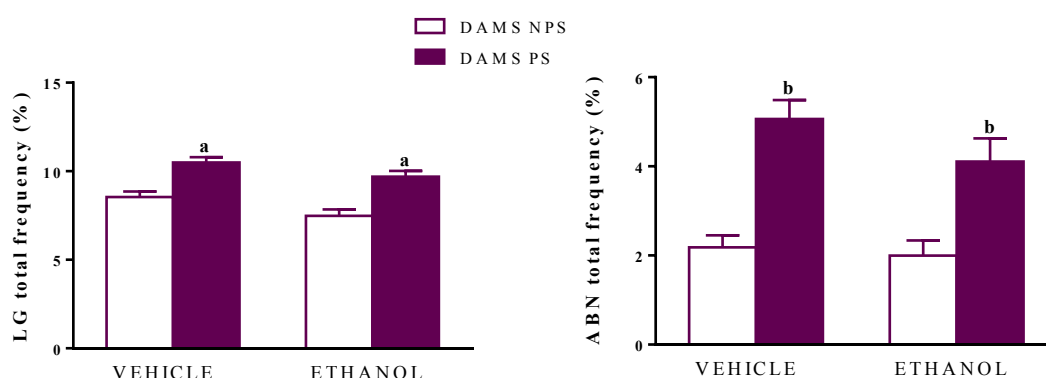

Figure 14. Rats were divided into 4 experimental groups: non pups separated Dams treated with vehicle (Dams VEH NPS), pups separated Dams treated with vehicle (Dams VEH MPS), non pups separated Dams treated with ethanol (Dams EtOH NPS) and pups separated Dams treated with ethanol (Dams EtOH PS). Data are means ± SEM of values from seventeen to twenty rats. [a]p<0.01 vs respective NMS control, [b]p<0.01 vs vs respective NMS control. All data were analyzed with a two-way ANOVA followed by Newman–Keuls post-hoc test.

5. *Effects of ethanol exposure during late pregnancy on plasma levels of corticosterone in Dams subjected to pup separation*

In order to assess if dams and maternal behavior could be related to changes in CTS level, we measure the plasma levels of CTS in dams after weaning (Figure 15).

The content of corticosterone was significantly lower (-42%) in dams that underwent pup separation (PS) (Dams VEH PS and Dams EtOH PS) and also in dams that received only ethanol during the last week of pregnancy (Dams EtOH NPS) (p<0.01). No significant difference was observed between dams EtOH NPS and Dams EtOH PS, which display the same pattern. ANOVA revealed a significant effect of ethanol prenatal treatment [F(1, 56)= 7,2224, p=0.009448] and maternal separation [F(1, 56)= 6,3765 p=0.014422]. For interaction between factors, ANOVA revealed a significant effect between ethanol prenatal treatment and maternal separation [F(1, 56)= 4,9919, p=0.029475].
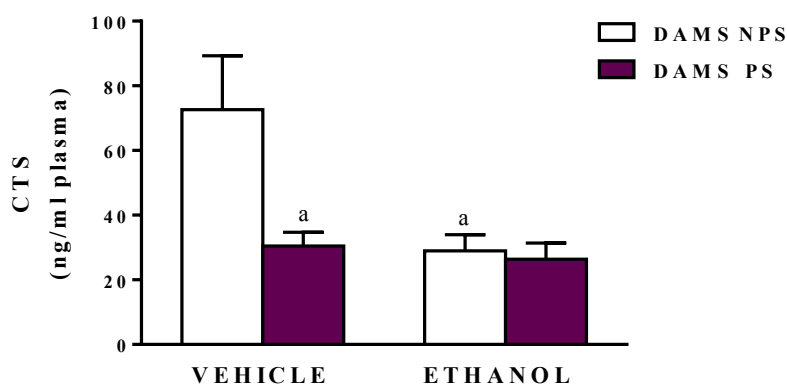

Figure 15: Rats were divided into 4 experimental groups: non pups separated Dams treated with vehicle (Dams VEH NPS), pups separated Dams treated with vehicle (Dams VEH PS), non pups separated Dams treated with ethanol (Dams EtOH NPS) and pups separated DAMS treated with ethanol (Dams EtOH PS). Data are means ± SEM of values from seventeen to twenty rats. [a]p<0.05 vs Dams VEH-NPS. All data were analyzed with a two-way ANOVA followed by Newman–Keuls post-hoc test.

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6. *Ascending Voluntary Ethanol Consumption Paradigm*

To evaluate the susceptibility to alcohol in offspring experimental groups, I measured the ethanol consumption using the ascending paradigm associated with a free choice (***Goodwin and Amit, 2000; Martinetti et al., 2006***).

Furthermore, I decided to set up ethanol consumption protocol into two different moments of offspring lifespan: in adolescent and adult animals, at 30 and 90 days of the age, respectively.

Ethanol consumption in adult rats

Figure 16 A-B shows the ethanol consumption, expressed in ml/Kg and g/Kg, respectively, in adult rats. As shown in the graph, there are no significant differences in the amount ml/Kg of ethanol consumed (A) between all the experimental groups for any ethanol concentration solution. Indeed a similar trend in ethanol consumption gr/Kg (B) was found in each experimental group. Ethanol consumption tends to decrease with increasing concentrations of ethanol (6-20%).
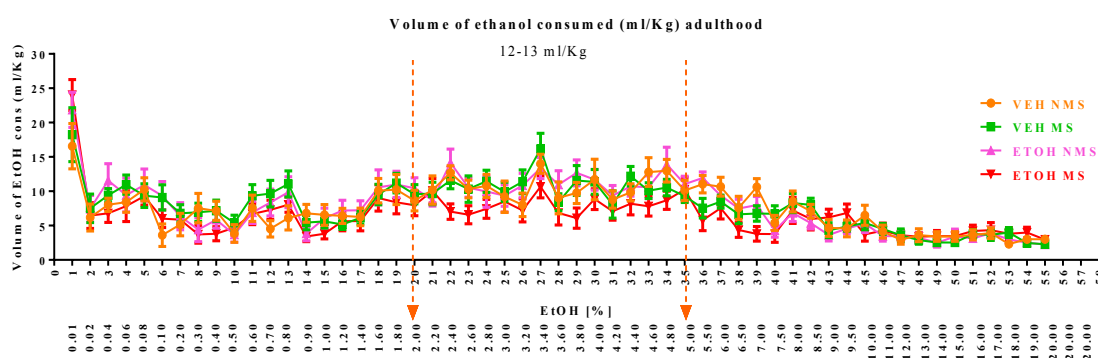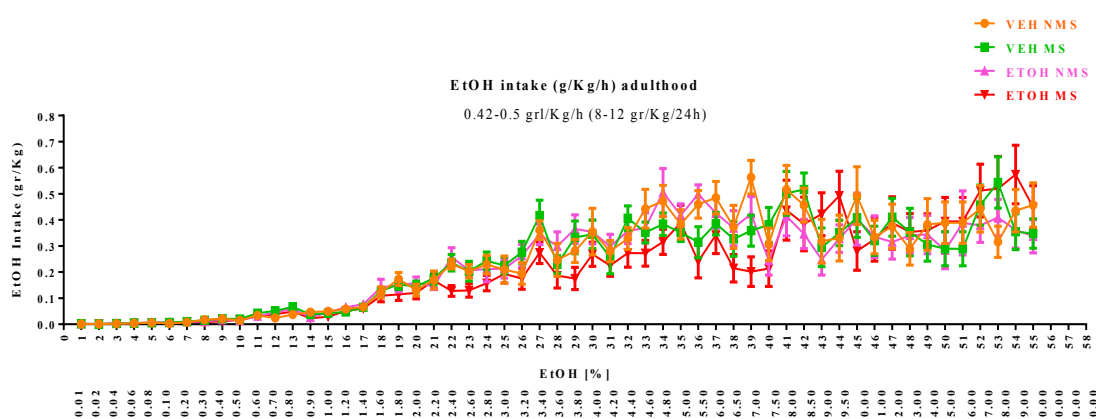

Figure16A: Effect of prenatal exposure to a low dose of ethanol (1g/Kg) and early maternal separation on ethanol consumption (mL/Kg) in the ascending voluntary consumption paradigm adult rats. Data are means ±SEM of values from 10 rats for experimental group, and were analyzed with a Repeated Measure (ANOVA) followed by Newman-Keuls post hoc test

Figure 16B: Effect of prenatal exposure to a low dose of ethanol (1g/Kg) and early maternal separation on ethanol consumption (g/Kg) in the ascending voluntary consumption paradigm in adult rats. Data are means ±SEM of values from 10 rats for experimental group, and were analyzed with a Repeated Measure (ANOVA) followed by Newman-Keuls post hoc test

With regards to the preference values in adulthood (Figure 17), there was a similar trend in all of the four experimental groups. Starting from 1% ethanol concentration up to 6.5% ethanol concentration, there is a significant preference (90%) towards ethanol in each groups. This preference then decreased in a uniform manner, remaining around 50% between 8% and 20% ethanol concentration.
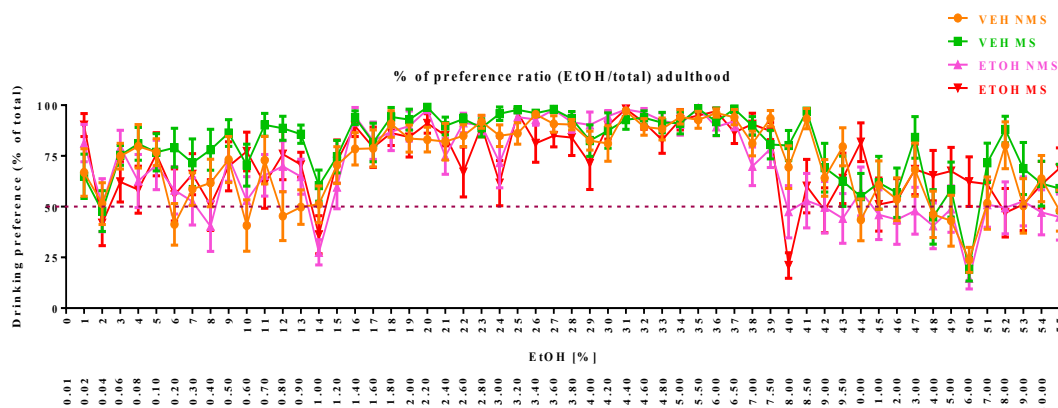
Figure 17: Effect of prenatal exposure to a low dose of ethanol (1g/Kg) and early maternal separation on preference % in the ascending voluntary consumption paradigm in adult rats. Data are means ±SEM of values from 10 rats for experimental group, and were analyzed with a Repeated Measure (ANOVA) followed by Newman-Keuls post hoc test

In order to emphasize the preference between tap water or ethanol solution data of each experimental group was divided into 4 graph (Figure 18A-B-C-D). Graph A shows the preference between tap water and ethanol in adult offspring VEH NMS. Starting from 1.2% up to 7% ethanol concentration graph shows a significant preference (80-90%) towards ethanol solution. Preference for ethanol solution is no significant different at the end of treatment using 8-20% of concentration.

In the offspring VEH MS group, preference for the ethanol solution is immediately evident, starting from very low concentration (0.04%) (B), and it is maintained significant up to 8% concentration (75-100%). The preference for ethanol shows a similar trend in offspring VEH NMS rats.

Similar considerations as offspring VEH NMS may be done for offspring EtOH NMS and EtOH MS. As shown in graph C-D, preference towards ethanol solution is evident starting from 1.2% concentration, to keep significant up to 6% concentration.
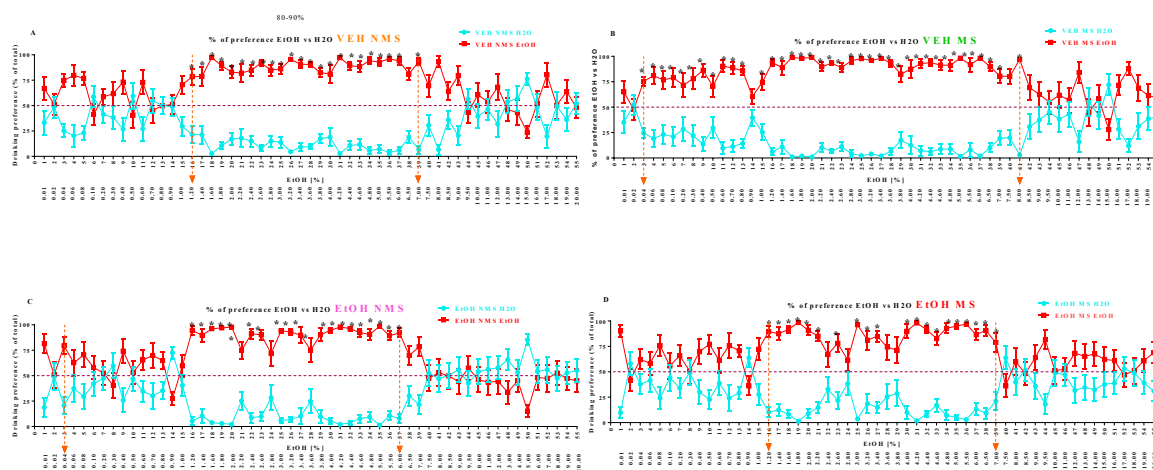
Figure 18: Effect of prenatal exposure to a low dose of ethanol (1g/Kg) and early maternal separation on preference % in the ascending voluntary consumption paradigm in adult animals, calculated for single experimental group. Data are means ±SEM of values from 10 rats for experimental group, and were analyzed with a Repeated Measure (ANOVA) followed by Newman-Keuls post hoc test *p<0.001 vs 50% of total fluid

This experiment shows a clear preference behaviour towards the ethanol consumption in VEH MS group already from the third day of drinking. However, the global AVOVA for consumption (ml/Kg) and intake (gr/Kg) did not show significant differences between all groups. So I calculated the mean of consumption and intake of three different ethanol concentration with range: 0.1-1%, 3-5% and 10-15%. From this analysis the ANOVA showed that there was a significant increase in ethanol consumption and intake of VEH MS already during the range 0.1-1% (Table1). These data, confirm the data showed in the figure 18B.

range	group	ml/kg	g/kg	% drinking preference
0.1-1%	VEH NMS	5.88 ± 0.504	0.023 ± 0.00244	56.52 ± 3.407
	VEH MS	[a]7.65 ± 0.491	[a] 0.033 ± 0.0028	*78.51 ± 2.665
	EtOH NMS	6.47 ± 0.55	0.028 ± 0.003	56.55 ± 3.546
	EtOH MS	[b]5.33 ± 0.384	[b] 0.023 ± 0.002	*63.58 ± 3.526
3-5%	VEH NMS	10.38 ± 0.574	0.32 ± 0.018	*86.89 ± 2.004
	VEH MS	10.46 ± 0.532	0.312 ± 0.016	*89.19 ± 2.037
	EtOH NMS	11.45 ± 0.52	0.344 ± 0.017	*91.79 ± 1.628
	EtOH MS	[c]7.81 ± 0.47	[c] 0.238 ± 0.015	*83.47 ± 2.594
10-15%	VEH NMS	3.98 ± 0.37	0.37 ± 0.033	50.14 ± 4.833
	VEH MS	3.920 ± 0.35	0.348 ± 0.029	55.44 ± 5.305
	EtOH NMS	4.61 ± 0.51	0.338 ± 0.030	41.17 ± 4.622
	EtOH MS	3.87 ± 0.37	0.35 ± 0.032	*61.24 ± 4.827

Table 1: Mean volume of ethanol solution consumed (mL/Kg) (A), ethanol intake (gr/Kg) (B), and drinking preference (ethanol/total fluid consumed during the 1-hour access period) (C), in the ascending voluntary consumption paradigm in adult rats that began the protocol at 90 PND. Each column is the mean of different ethanol concentration range (0.1-1%, 3-5%, 10-15%). Data are means ±SEM of values from 10 rats for experimental group, (two-way ANOVA followed by Newman–Keuls post-hoc test). *p<0.001 vs 50% total fluid. .[a]p<0.05 vs VEH-NMS; [b]p<0.05 vs VEH-MS; [c]p<0.05 vs VEH-MS, EtOH-NMS

Ethanol consumption in adolescent rats

Figure 19A-B show the consumption, expressed in ml/Kg and g/Kg respectively, in adolescent rats subjected to 1hour free access ascending ethanol concentration paradigm. The graph above describes that each experimental group shows similar trend in the volume of ethanol consumed, starting from lower concentrations of ethanol (0.01% to 1.8%). Differences are highlighted in the concentrations range between 2% and 6%, in which offspring EtOH MS showed a significant average consumption of 2-10 ml/Kg (0.4-0.5 gr/Kg), versus average consumption of 15-18 ml/Kg (8-12 gr/Kg) showed by offspring VEH NMS.
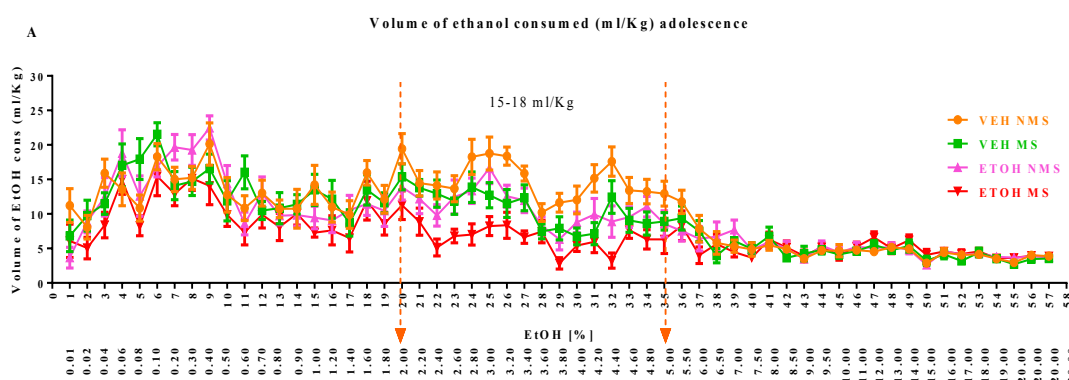

Figure 19A: Effect of prenatal exposure to a low dose of ethanol (1g/Kg) and early maternal separation on ethanol consumption (mL/Kg) in the ascending voluntary consumption paradigm in adolescent (A) and adult (B) animals. Data are means ±SEM of values from 10 rats for experimental group, and were analyzed with a Repeated Measure (ANOVA) followed by Newman-Keuls post hoc test
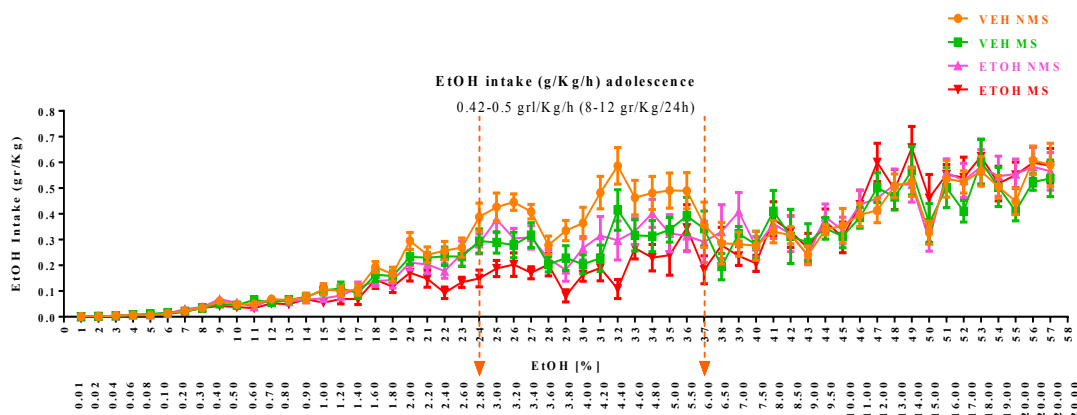
Figure 19B: Effect of prenatal exposure to a low dose of ethanol (1g/Kg) and early maternal separation on ethanol consumption (g/Kg) in the ascending voluntary consumption paradigm in adolescent (A) and adult (B) animals. Data are means ±SEM of values from 10 rats for experimental group, and were analyzed with a Repeated Measure (ANOVA) followed by Newman-Keuls post hoc test

Furthermore, I wanted to assess whether rats subjected to both stressors or just one of them had a preference for ethanol or water. In Figure 20 is shown the preference expressed as a percentage between ethanol consumption and or tap water vs total fluid, in rats at 30 days of the age. In the lower concentrations of ethanol (from 0.01% up to 1.4%) preference values are between 30% and 70%, but there are no significant differences and the trend is similar for the four experimental groups. The significant preference of ethanol concentration started at 2,4 % up to 6%, in which we VEH NMS shows a strong preference (68% -90%) to the ethanol solution, compared to the other 3 experimental groups. These values % decrease considerably to below 50%, highlighting the preference for the bottle containing tap water.
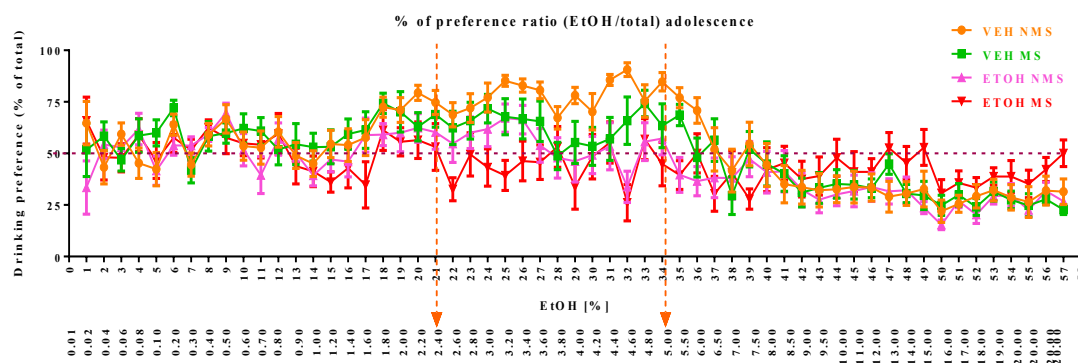
Figure 20: Effect of prenatal exposure to a low dose of ethanol (1g/Kg) and early maternal separation on preference % in the ascending voluntary consumption paradigm in adolescent rats. Data are means ±SEM of values from 10 rats for experimental group, and were analyzed with a Repeated Measure (ANOVA) followed by Newman-Keuls post hoc test

We then next measured the preference between tap water and ethanol for each of the four experimental groups in adolescence (Figure 21). In graph A is shown the preference towards ethanol in VEH NMS in which the significant preference for ethanol bottle starts at 1.6% concentration up to 5.5% of concentration, preference is significantly higher for the ethanol solution (80%-90%). This trend of preference is completely inverted at higher concentration of ethanol (9-20%). In other groups VEH MS and EtOH NMS (graph B-C), trend is different than VEH NMS. Here, is not a significant preference for the ethanol solution, even if a tendency to prefer ethanol solution is shown in concentrations between 1.4% and 3.2% (60-65%). As showed in the previous experimental group, starting from 9% of ethanol concentration the trend was completely inverted and there was a significant preference for tap water until the end of treatment.

Finally, graph D shows the trend of preference in EtOH MS. For the entire treatment, there was not a significant preference towards ethanol solution. Graph, shows that the trend is largely the same for all ethanol concentrations and there was no preference for any solution (tap water and ethanol).
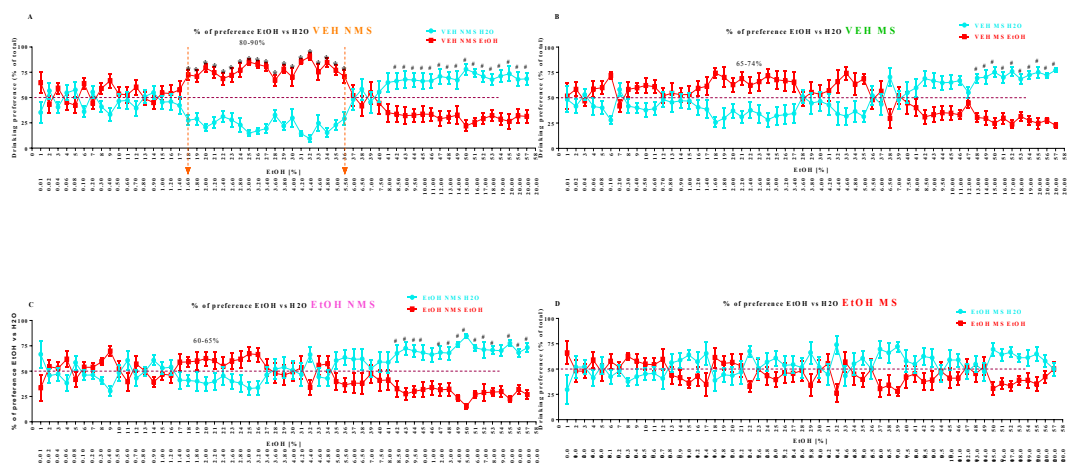
Figure 21: Effect of prenatal exposure to a low dose of ethanol (1g/Kg) and early maternal separation on preference % in the ascending voluntary consumption paradigm in adolescent animals, calculated for single experimental group. Data are means ±SEM of values from 10 rats for experimental group, and were analyzed with a Repeated Measure (ANOVA) followed by Newman-Keuls post hoc test *p<0.001 vs H2O; #p<0.001 vs EtOH

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Also for adolescent rats experiment I calculated the mean of ethanol consumption and intake at 0.1-1%, 3-5% and 10-15% range. From this analysis the ANOVA showed that there was a significant increase in ethanol consumption and intake of VEH NMS during the range 3-5% (Table 2).

range	group	ml/kg	g/kg	% drinking preference
0.1-1%	VEH NMS	14.3 ± 0.852	0.0561 ± 0.0043	54.97 ± 2.092
	VEH MS	14.22 ± 0.756	0.05382 ± 0.0038	56.77 ± 2.235
	EtOH NMS	14.19 ± 0.757	0.0514 ± 0.00326	52.39 ± 1.951
	EtOH MS	[a]10.89 ± 0.624	[a]0.0405 ± 0.0028	51.43 ± 2.193
3-5%	VEH NMS	14.36 ± 0.579	0.4302 ± 0.017	*79.32 ± 1.631
	VEH MS	[b]9.533 ± 0.532	[b]0.285 ± 0.016	*62.27 ± 2.749
	EtOH NMS	[b]10.23 ± 0.577	[b]0.303 ± 0.0169	52.01 ± 2.686
	EtOH MS	[c]6.317 ± 0.449	[c]0.189 ± 0.01398	44.86 ± 2.857
10-15%	VEH NMS	4.488 ± 0.242	0.4217 ± 0.0222	*30.27 ± 2.788
	VEH MS	4.592 ± 0.271	0.4331 ± 0.0273	*33.07 ± 2.277
	EtOH NMS	4.565 ± 0.265	0.426 ± 0.044	*28.00 ± 2.407
	EtOH MS	5.237 ± 0.333	0.498 ± 0.0333	43.95 ± 3.252

Table 2. Mean volume of ethanol solution consumed (mL/Kg), ethanol intake (gr/Kg), and drinking preference (ethanol/total fluid consumed during the 1-hour access period), in the ascending voluntary consumption paradigm in adolescent rats that began the protocol at 30 PND. Each column is the mean of different ethanol concentration range (0.1-1%, 3-5%, 10-15%). Data are means ±SEM of values from 10 rats for experimental group, (two-way ANOVA followed by Newman–Keuls post-hoc test). *p<0.001 vs 50% total fluid. .[a]p<0.05 vs VEH-MS, EtOH-NMS, [b]p<0.001 vs VEH-NMS, [c]p<0.001 vs EtOH-NMS.

Discussion

   The results obtained in this study show that the association of two stressful events, such as prenatal ethanol exposure and maternal separation during the first weeks of life, leads to distinct changes in the neuroendocrine response to acute stress as well as emotional state and susceptibility to ethanol consumption in adult male rats.

It is reported from several groups that an acute stress results in a significant increase in AP plasma levels in control animals (***Barbaccia et al., 1996; Purdy et al., 1991***) through HPA axis activation. Accordingly, also acute treatment with ethanol, that leads to an activation of the HPA axis, is considered a stressful event, capable of increasing AP in rat brain and plasma (***Morrow et al., 1999***). This effect is observed at low to moderate doses of ethanol (1-2g/kg). In our experiments, prenatal exposure to moderate doses of ethanol (1g/kg) administered during the last week of gestation, does not change significantly the plasma levels of AP in the adult offspring rats. On the other hand, the association of prenatal ethanol exposure and maternal separation leads to a significant reduction in AP basal levels, an effect similar to that previously shown by social isolation chronic stress (***Serra et al., 2000***). In previous studies, animals subjected to maternal separation did not show altered sensitivity to the steroidogenic effect induced by acute foot-shock stress on AP and CTS plasma levels (***Biggio et al., 2014***). In contrast, in the present study the association of prenatal exposure to ethanol and maternal separation causes an increased response of the HPA axis to acute foot-shock stress, resulting in higher levels of plasma CTS and AP, compared to control animals.

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Here, we show that maternal separation alone or in association with a prenatal alcohol exposure leads to a significant decrease of CTS basal level in plasma, without changes in AP levels of VEH-MS group. This may be due to the fact that all dams have been subject to intragastric administration (IGA) of ethanol, which is in itself an extremely stressful event, despite the habituation period to which the animals were subjected (***Kelly et al., 2008***). Therefore, IGA could be the responsible of the decrease in CTS basal levels.

When the animals are exposed to acute foot-shock stress, our results show an increase in CTS blood levels in all the experimental groups compared to counterparts not subjected to foot-shock stress. Successive analysis show that the increase in the percentage of CTS in VEH-MS is more pronounced than the increase of plasma CTS levels in EtOH-MS group (224.5 ± 24.29 % vs VEH-NMS). These results, in agreement with previous reports (***Zimmemberg and Brown, 1998***), suggest that these animals are more sensitive to an acute stressful stimulus, and supports the hypothesis that adverse events experienced during the prenatal period and immediately after birth have long-term consequences in response to stressful experiences during adult life (Fig. 12).

This increase in stress hormone (AP and CTS) levels in EtOH-MS group is associated with anxiety behavior, demonstrated by a significant reduction in the open arms entries in the Elevated Plus Maze test. The lack of difference in the total entries in the elevated plus maze test between all the experimental groups suggest that there are no significant changes in locomotor and exploratory activity. Thus, the decrease in open arms entries is probably due to the interaction between pre and postnatal stress. Therefore, stressful events that occur during critical periods of brain development, such as prenatal period and infancy alter the emotional state and stress response in adulthood. These findings are also consistent with previous report from our laboratory, showing that in an animal model of chronic stress,

induced by the social isolation, the reduction of AP basal levels is associated with a state of conflict, a reduction of the GABAergic transmission and greater sensitivity to the steroidogenic effect induced by stress *(Serra et al., 2000)*.

Allopregnanolone is one of the most potent positive modulators of the GABAergic transmission (***Biggio and Purdy, 2001***). Thus, the reduction of AP observed in EtOH-MS group under basal condition would be consistent with alterations of the GABAergic transmission and emotional state. The increase in AP levels following exposure to acute stress is part of a compensatory mechanism to restore the reduced GABAergic transmission induced by chronic exposure to stress (***Barbaccia et al., 1996; Concas et al., 1998; Drugan et al., 1989; Biggio et al., 1990***). Consistent evidences suggest a significant correlation between emotional state and stress response in adulthood and the quality of maternal care received in early life. (***Caldji et al., 1998; Van Hasselt et al., 2012; Liu, 1997)*.

Maternal care has a major role in the development of the offspring, and could affect the psychobiology of the individual. Therefore, I wanted to assess whether, in animals subjected to the association of pre and postnatal stress, an altered HPA axis responsiveness was due to a different maternal behavior pattern. As expected (***Biggio et al., 2014***), maternal separation induced an increase in arched back nursing and in pup licking frequency (***Myers et al. 1989; Levine, 1994***), two of the most affective maternal behaviors. These results indicate that dams exposure to low dose of ethanol during the last week of pregnancy do not show changes in the quality of maternal behavior, suggesting that alteration in emotional state and the increased stress response in animals subjected to prenatal ethanol exposure and maternal separation is not related to the quality of maternal care.

It is well known that, besides genetic factors, environmental influences during brain development play a pivotal role in the development of vulnerability to ethanol consumption.

Epidemiological studies showed that alcohol consumption in humans begins in early adolescence and the amount consumed tends to increase during adolescence and then fall into adulthood (***Merikangas, 1990***). In order to find whether this trend in humans is due to social reasons rather than to biological basis, I measured the consumption and preference to ethanol in a group of adolescent and adult rats prenatally exposed to ethanol and subjected to maternal separation during the first weeks of life. In this study, access to ethanol was given from post natal day 30, during adolescence, and from post natal day 90 during adulthood in order to examine whether the effects of early environmental factors on vulnerability to ethanol differ in rats subjected to the same procedure (prenatal ethanol exposure and maternal separation) in different moments of life. For this purpose, I used the Ascending Voluntary Ethanol Consumption Paradigm (***Martinetti et al., 2006***).

In literature there are many conflicting data on the predisposition to alcohol consumption after exposure to maternal separation. Some authors report predisposition to alcohol consumption after maternal separation (***Huot et al., 2001***) while other showed a lack of this propensity (***Roman et al., 2004)***. The reasons for the presence of such conflicting data are due to the different protocols, as well as to the different strains of rat and mouse examined (***Nylander et al., 2013***). Sprague Dawley is a rat strain that usually doesn't drink easily compared to preferring rats (***Martinetti et al., 2006***), so it is a good rats model to study the really effect of stress causing vulnerability in alcohol free-access assumption. All experimental groups of adult Sprague-Dawley rats used in this work showed a clear preference for ethanol, resulting in a lack of difference among groups between 1.40% and 7% (Fig. 17). However, there is an increased consumption of ethanol in the VEH MS group, already significant at very low concentrations, between 0.1 and 1% when compared to controls. (Table 1). Moreover, this group subjected to maternal separation shows a clear preference for ethanol respect to water

since the first sessions. These data are in accordance with other results, that demonstrating high tendency for ethanol intake in a free-choice paradigm between water and ethanol after exposure to a long maternal separation (***Gustafsson and Nylander, 2006***).

In our work, besides the susceptibility to ethanol consumption in adulthood, we also wanted to assess what happens during adolescence, an important period for the vulnerability to drugs of abuse. Our data show that there is no significant difference between the four experimental groups. Furthermore, in the 3-5% range of ethanol concentrations, the VEH-NMS group shows an increase in alcohol consumption, not present in the other experimental groups (Fig. 21A)

The lack of interaction between pre- and postnatal stress in terms of ethanol consumption is, probably due to the fact that in utero the olfactory system of the fetus is not yet mature, thus it cannot work properly to detect ethanol. The limited EtOH consumption observed in adolescent animals prenatally exposed to ethanol and subjected to early maternal separation (Fig. 21) could be due to an aversive reaction to ethanol, developed during the ethanol intragastric administration in dams in the last stages of pregnancy (***Pepino et al., 1998; 1999; 2001; 2002; Molina et al., 2000***). Interestingly, this limited drinking behavior is not observed in adult animals (Fig. 18), probably due to the loss of aversive reaction to ethanol (defense mechanisms) present during adolescence. Furthermore, these animals probably came out from two stressful closely events differently from adults that have had much more time to recovery. This could partially explain the different attitude of offspring to ethanol consumption.

Further studies are needed to understand the molecular mechanisms behind these age differences.

Altogether our data suggest that stress occurring during the prenatal period, associated with a postnatal stress, leads to an altered emotional state in animals. Moreover, the association of the two stressors (prenatal ethanol treatment and maternal separation) determines a preference for ethanol consumption in adult period but not during adolescence, suggesting the presence of molecular mechanisms that protect adolescents from a consumption behavior.

These findings may help to better understand the importance of the interaction of different type of stressful stimuli suffered early in life onto behavioral and molecular aspects in adulthood.

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