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Neonatal Maternal Separation and Alcohol Abuse in C57BL/6J mice: A Study of the Functional Alterations of GABAergic and GLUergic Systems and the Possible Protective Role of Esterogen

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Presentata da: **Dott. Ashish Avinash Gorule**

Coordinatore Dottorato: **Prof. Walter Fratta**

Tutor: **Prof. Enrico Sanna**

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INTRODUCTION

The term “stress”, was coined by Hans Selye in 1936, who defined it as “the non-specific response of the body to any demand for change”. Selye had reported in numerous experiments that laboratory animals subjected to acute but different noxious physical and emotional stimuli (blaring light, deafening noise, extremes of heat or cold, perpetual frustration) all exhibited the same pathologic changes of stomach ulcerations, shrinkage of lymphoid tissue and enlargement of the adrenals. He later demonstrated that persistent stress could cause these animals to develop various diseases similar to those observed in humans, such as heart disease, kidney disease, etc.

The magnitude of the stress response depends on the stressor stimulus, as the body's response will be appropriate to the level of stress. Beyond stress we suffer every day, there is also stress that can induce permanent responses in our bodies; this is mostly done in certain periods of our lives, and precisely during development. In fact stress suffered during the neonatal period can result in changes of some physiological functions that regulate growth, metabolism, reproduction and immune response. Studies have shown that this type of stress can induce behavioural changes in adulthood and can increase the risk of developing psychiatric disorders such as depression (Kendler *et al.*, 2002) and psychosis (Morgan *et al.*, 2007).

Both animals and humans share similar characteristics in the expression of emotions and this may increase the possibility to study the mechanisms underlying stress responses using animal models have been created different experimental models in laboratory animals in order to study the different behavioural responses to various stressful stimuli, the restraint stress, the foot shock and the forced swim stress. But to evaluate the extent of emotional states such as anxiety using elevated plus maze, the light-dark box, the social interaction test, open field test, the novelty suppressed forced swim test, and the Vogel test.

The experiences during childhood can influence in a sometimes irreversible the proper development of neurobehavioral and brain function observable even in adulthood, both the

environment and maternal care received in the first few days of life markedly affect the brain development of the baby. Positive or negative stimuli, received during the early stages of life, can cause neurobiological changes and long-term behavioural, involving neuronal processes not yet well characterized that, when altered, due to adverse events suffered during the perinatal period, they may have effects on the risk of developing behavioural disorders in several animal species, such as primates and rodents.

In rodents, the protocol of repeated maternal separation (RMS), which is experimentally obtained by separating the newborn from its mother for a variable number of hours (usually 3-6h) daily, from 1^{st}-3^{rd} till 14^{th}-17^{th} day of life, is frequently used to examine the effects of different types of stress suffered in perinatal age. This type of stress induces persistent changes in psychological, neurobiological, and emotional changes leading to lasting changes of the hypothalamic-pituitary-adrenal axis (HPA axis) (Ellenbroek *et al.*, 1998; Enthoven *et al.*, 2008 ; Lyons *et al.*, 1998; Pryce *et al.*, 2005) and it was also demonstrated how different types of stress are associated with increased vulnerability to abuse substances (Koob and Kreek 2007; Shina 2001). These findings have allowed to suggest a close correlation between stress, neural circuitry of reward (Yap and Miczek 2008) and regulation of the HPA axis that is crucial in hormonal responses to stress. In fact, several studies have shown a positive association between stress and increased alcohol consumption among alcoholics and social drinkers (Dawson *et al.*, 2005; Kaufman *et al.*, 2007; Linsky *et al.*, 1985); stress patterns as restraint stress in rats (Bowers *et al.*, 1997; Overstreet *et al.*, 2007) and repeated social defeat stress in mice (Croft *et al.*, 2005) result in an increase in alcohol consumption; other models such as repeated swim stress instead are associated with a decrease in the consumption of alcohol, depending on the animal model used (Boyce-Rustay *et al.*, 2008).

The protocol of the RMS was considered to be one of the most effective in inducing neonatal stress (Levine 1967; Levine 2001; Wigger and Neumann 1999), producing consequences

both neuronal behaviour, including vulnerability to alcohol consumption and drug abuse in adulthood (Gilmer and McKinney 2003; Moffet *et al.*, 2007; Sanchez *et al.*, 2001). During the neonatal period in fact, adequate maternal care are essential for the formation and regulation of the HPA axis and the stress response (Levine 2005). This period of life is characterized by a low responsiveness of the HPA system to stress that seems to be caused, in part, by a reduced sensitivity of the adrenal gland for the ACTH (Rosenfeld *et al.*, 1992), resulting in an amplification of the negative feedback induced by corticosterone (CORT) (Walker *et al.*, 1986). It has been shown that the effects induced by maternal separation on alcohol are generally gender-dependent, concentration-dependent and time-dependent (Gustafsson *et al.*, 2005; Gustafsson *et al.*, 2007; Gustafsson and Nylander 2006; Marmendal *et al.*, 2004; Roman *et al.*, 2004; Roman *et al.*, 2005).

Applying the protocol of RMS it is foundamental the timing of separation since it has bben well demonstrated that short vs long time of separation can induce oppsit effects (Ploj *et al*., 2003). For example, maternal separation lasting 15min (MS15) reproduces what naturally occurs when the mother goes to search food (Grota and Alder 1969). When we want to study the developmental changes, instead of negative conditions, then the duration of the separation from the mother increases; a duration of 180-360min stops in fact the interaction between mother and pups. It has been shown that a prolonged separation is associated with hyperactivity of the HPA axis while that of short duration to a hypoactivity of the same axis (Hout *et al.* 2001; Levine 1967; Meerlo *et al.*, 1999; Plotsky and Meaney 1993). Weininger (1999) has observed and demonstrated how handling, the act of gently caress the pups for 10min every day for three weeks following the birth, turns out to be positive and have a dramatic effect on behaviour assumed then in adulthood. In addition, the pups separated from the mother for 8h but not separated from each other. Home-separated rats, in which pups remained in the home cage, are much more susceptible to stress than novelty-separated rats, in which pups were placed individually in a novel cage (Daskalakis *et al.*, 2011). As shown in various researches, the RMS 60-180min conditions of home-separated rats it is

associated with an increased consumption of ethanol or preference in free choice between water and ethanol (Hilakivi-Clarke *et al.*, 1991). Under conditions of novelty-separated rats, no difference was observed between MS15 and MS180 in ethanol consumption (Oreland *et al.*, 2011). All these studies show that the effects of the RMS on the HPA axis.

Weigger et. al. reported that in male and female rats, periodic maternal separation causes chronic changes in emotional behaviour and HPA axis activity. The effects of the RMS on emotional behaviour are in fact more pronounced in males than in females, and are associated with an increased release of ACTH in response to the test elevated plus maze; unlike forced swimming test that instead did not differ between males and females. In addition, the RMS180 determines the appearance of behaviours associated with anxiety much more pronounced in the male, showing greater vulnerability HPA male to maternal separation (Wigger *et al.*, 1999).

Ploj and his collaborators have shown that male Wistar rats, the MS15 causes a decrease in consumption of ethanol in adulthood, while the separation for longer periods (MS360) has the opposite effect and thus stimulate consumption (Ploj *et al.*, 2003). Females instead show a different behaviour than males. In fact, the results obtained with either MS15, MS360 and in control conditions are the same. No observed was difference on ethanol consumption and even on the preference between water and ethanol (Roman *et al.*, 2003). The mechanisms that explain the differences gender on ethanol consumption after maternal separation are still unknown, but it is thought that could be due both to hormonal differences and also to the fact that the mother interacts differently with puppies of the opposite sex (or a combination of these factors). It has been shown that maternal separation induces neurochemical alterations in several endogenous systems (Anand and Scalzo 2000; Newport *et al.*, 2002; Papaioannou *et al.*, 2002), such as on the opioid system, and how the effects in long-term maternal separation on this system are greater in males than females (Ploj *et al.*, 1999, 2000). In some studies, it has been reported as the different stages of the estrous cycle in female does not influence the different consumption of ethanol, demonstrated by the fact

that the consumption of ethanol has been evaluated in several days and thus including all stages of the estrous cycle (Roman *et al.* 2005). A study conducted on mice showed that ethanol, as well as psychostimulants and morphine, can induce behavioural changes, such as change in locomotion after repeated administration (Fish *et al.*, 2002; Masur *et al.*, 1980; Masur *et al.*, 1986; Phillips *et al.*, 1997). Also, in this case it was shown that maternal separation induces gender-dependent effects. No effect on the development of this alteration of locomotion was observed in males, but shows effect in females subjected both short (MS15) that long (MS180) maternal separation. Although it appears more quickly in terms of MS180. Moreover, females subjected to MS180 show an increase of basal levels of corticosterone (CORT) in plasma in comparison to the females subjected to MS15, this difference does not seem to be associated with greater rate of appearance in alteration of behaviour in females undergoing MS180. In males instead, both MS15 and MS180, there is an increase in the levels of CORT in response to the administration of ethanol but no effect on alteration locomotion. These results do not exclude the influence of CORT behaviour, which could be analyzed with the adrenalectomy and administration of CORT at different concentrations (Kawakami *et al.* 2007).

Lehmann and colleagues analyzed the behaviour of the adult both male and female Wistar rats, when subjected to maternal separation of 24h in the postnatal day 4 (MS4), 9 (MS9) and 18 (MS18). Again were observed gender differences, as the effects of maternal separation they are more pronounced in males, especially when assessed in tests used to determine the anxiety and fear; MS4 is the cause of the deficit more pronounced in males on tests of learning (including conditioned freezing) while MS9 increases the response in males accounted for using the water maze test. All this shows that the MS, regardless of age of separation, has different effects depending on the type of test used and that males are more sensitive to stress than females and therefore more susceptible to maternal separation (Lehmann *et al.*, 1999).

The work was done on C57BL/6 mice, where it was evaluated anxiety and behaviours associated with fear, are modulated by maternal separation. It has been proven with tests such as open field and elevated plus maze that males separated from her mother for three hours a day, from postnatal day 1 to 14 (PND1-14), show levels of anxiety and fear more than controls (Romeo *et al.*, 2003). It had been shown that these animals show changes on the release of CRH by the cells that are involved in mediating the stress response (Meaney, 2001), for example, the mCRH and the consideration peptide are increased in the core of the amygdala, in hypothalamus, and locus coeruleus as well as levels of CRH receptors in the raphe nuclei and locus coeruleus (Ladd *et al.*, 1996; Meaney 2001; Plotsky and Meaney 1993).

Romeo and his collaborators also observed females subjected to maternal separation for 3h a day from PND1-14. They have been tested both in the phase of the estrous cycle diestrus (when estrogen levels in the blood are low) and estrus (when estrogen levels in the blood are high) to assess how the fluctuations of estrogen can affect the emotions associated with maternal separation. In fact shown reduced levels anxiety and fear only when it is in the diestrus phase of the estrous cycle and only in the open field test (Romeo *et al.*, 2003), confirming the role of estrogen as a modulator of anxiety and fear (Morgan and Pfaff 2001, 2002) and how to induce hippocampus synaptogenesis (Kretz *et al.*, 2010).

Estrogen

Estrogen is a steroid hormone that regulates neuronal excitability, mood and emotions. Modulates cognitive functions, has neuroprotective effects (from tone to neurons), modulates pain perception, motor coordination rule. At the central level it is located in the hypothalamus, midbrain, cerebral cortex, hippocampus, amygdala, grey matter and the septum. Is then released both peripheral and central level after the activation of hypothalamic-pituitary-gonadal axis. In the arcuate nucleus of the hypothalamus is released, intermittently, GnRH (gonadotropin releasing hormone), which is to stimulate the release of pituitary LH (luteinizing hormone) and FSH (follicle stimulating hormone), which stimulate the ovaries to produce steroids.

Estrogen is synthesized from cholesterol that is transported into the mitochondria by StAR protein also expressed in the hippocampus of males and females, indicating the ability of this tissue to produce de novo sexual neurosteroids (Wehrenberg *et al.*, 2001). The cholesterol is then transformed into pregnenolone and this then hydrolyzed in position 17 (17-OH pregnenolone); through the CYP17 it is converted to DHEA which is the precursor of androstenedione, which can be transformed into testosterone from a 17-β HSD. This first phase takes place in the theca cells of the ovary which surround the outside of the ovarian follicle. Subsequently testosterone passes in granulosa cells, internal follicle and by an aromatase (regulated by FSH) is formed on the 17 β-estradiol and, from this estriol or the estrone.

The action of estrogen is classically mediated by its binding to nuclear receptors (Klinge 2001) divided into two families, the receptors α and β, those that have a molecular structure similar (but the genetic origin is distinct), but also homodimer form heterodimers and are distributed differently. Three are known isoforms of receptor α and 5 of those β. They are more present in the pituitary, kidney, adrenal gland but also in the gonads, those β are found in the ovaries, uterus. At the central level are both widely distributed.

Some researchers have shown that probably the effects of estrogen are due also by binding to membrane receptors (Moss *et al.*, 1997; Revelli *et al.*, 1998; Razandi *et al.*, 1999; Toran-Allerand, 2002), initially called ER-X and which are mediating effects neuronal excitability, modulation of protein G, on increased cAMP via activation of the MAP-KINASE and the effects of calcium and potassium channels. These membrane receptors are present both centrally in dendritic spines and axons, but also in the periphery of the reproductive organs. One wonders if these membrane receptors are the same expressed at the nuclear level, whether they derive from the same gene, and if you actually are G protein-coupled (as they have the ability to modulate the G-protein), whether they are associated with MAP-KINASE or if they have an intrinsic kinase (Toran-Allerand 2013).

Prange-Kiel and his colleagues found that the GnRH receptors are abundantly expressed in the hippocampus than females and that, besides regulating the release by the ovaries are essential for the synthesis of estrogen level in hippocampus. Also, observed that the GnRH increases the formation of synapses in hippocampal cell cultures of females. It remains to understand what the GnRH can regulate the synthesis of neurosteroids in the hippocampus of male (Fester *et al.*, 2014).
It has been shown that the removal of gonads results in a reduction of dendritic spines in the hippocampus of males and females (Gould *et al.*, 1990; Leranth *et al.*, 2004); the recovery of these spines can be obtained with the injection of estradiol in females but not in males, in fact, in these last is the injection of testosterone to restore the loss of the plugs (Leranth *et al.*, 2004).

A recent publication showed that estrogen plays a key role in inducing synaptic plasticity. In hippocampal cell cultures using Letrozole, an aromatase inhibitor, and has been shown a significant reduction in the number of dendritic spines in the hippocampal CA1 region (Vierk *et al.*, 2014). All this supports the idea that estrogen, is essential for synaptogenesis (Prange-Kiel *et al.*, 2013). In hippocampus of male and ovariectomized female, estrogen concentration is extremely low. These differences are consistent with the gender difference on estrogen concentration in

plasma (Fester 2012). A recent study shown that the concentrations of estrogen in the hippocampus are correlated with the concentrations of estrogen in the blood during the the estrous cycle (Kato *et al.*, 2013). Vierk and his team then wanted to study the correlation on the loss of synaptic density induced Letrozole and LTP. Following a treatment of 7 days with Letrozole. The LTP was no longer present in the hippocampus of females, whereas in males LTP was reduced by 20% without any loss of synapses following treatment (Vierk *et al.*, 2014). These results are consistent with studies showing that estrogen induces an increase of LTP in the CA3-CA1 (Foy *et al.*, 1999; Kim *et al.*, 2006). The role of glutamate receptors in this context is not very clear but it has been shown that estrogen increases the NR1 subunit of NMDA receptors in females (Gazzaley *et al.*, 1996).

Many works has been reported that estrogen has a positive and beneficial effect on memory (Swerwin *et al.*, 1988; Philips and Swerwin 1992; Duka *et al.*, 2000; Maki *et al.*, 2001) and the risk of dementia (Yaffe *et al.* 1998; Nelson *et al.*, 2002). And it was shown that, in women treated with Letrozole are observed deficits of memory, a result in agreement with the fact that estrogen is also involved in the regulation of cognitive functions (Vierk *et al.*, 2014).

GABAergic transmission

The gamma-aminobutyric acid (GABA) was identified by E Roberts and S Frankel in 1950, in brain extracts of different animal species. Only a few years later it was suggested that the GABA had an inhibitory action at the level of the different species, Further studies allowed animals to classify the GABA as a amino acid that in the CNS of mammals has function of neurotransmitter inhibitor, that is a substance produced by nerve cells and from these liberated in the synaptic space to convey a message inhibitor. These studies demonstrated that the GABA is the most abundant inhibitory neurotransmitter in the brain of mammals, where about 35-40% of the GABAergic synapses appear to be more recent studies have also suggested that this amino acid has a significant role in the control of many brain functions and therefore in the pathophysiology of a number of mental illnesses and neurological disorders. The GABA is formed by decarboxylation of glutamic acid, this reaction is catalyzed by GAD, an enzyme which has as the cofactor pyridoxal phosphate. The distribution of GAD in SNC always reflects that of GABA; the enzyme is found in soluble form almost exclusively in the cytoplasm of nerve endings. The GABA synthesized in the cytoplasm, is stored in synaptic vesicles present in the terminal portion of the axons and is released either spontaneously or following nervous stimulation induced by depolarization Ca^{2+} dependant. Then there are specific mechanisms that remove quickly GABA uptake from the synaptic cleft thus ending its inhibitory postsynaptic These systems are present both in the nerve ending that on glial cells. The GABA is then subsequently degraded by the enzyme GABA-to-chetoglutaricotransaminasi (GABA-T), which deaminates to succinic semialdehyde; this is then oxidized to succinic acid by one-succinic semialdehyde dehydrogenase NAD-dependent joins the Krebs cycle.

The amino group is transferred from the GABA-T to a molecule of α-ketoglutaric acid to form glutamic acid which is reused for the synthesis of new GABA. Electrophysiology and biochemical studies have demonstrated the existence of two different binding sites on GABA

conventionally called: GABAA and GABAR. These differ in pharmacological profile, molecular structure and mechanism of signal transduction receptors. GABAA receptors are permeable to the Cl^- ion-channel, while GABAR receptors are G protein-coupled type of inhibitory receptors. The GABAA receptors are characterized by a high sensitivity to bicuculline and muscimol respectively. Selective antagonist and selective agonist have high affinity for the binding site of GABA, and contain specific binding sites for the benzodiazepines and barbiturates that modulate their function. GABAR receptors are activated selectively by the derivative of GABA, [3-p-chlorophenyl-GABA (baclofen) and unlike the GABAA receptors are insensitive to bicuculline and muscimol.

Since the Cl^- is the only ion permeating through the GABAA receptor, receptor activation fixing the membrane potential to that of equilibrium of Cl^-, which is normally -70mV. The activation of this receptor thus reduces neuronal excitability. The inhibitory response induced by the GABAA receptor occurs through pre- and post-synaptic mechanisms. The post-synaptic hyperpolarization is typical of cerebral neurons (cortical cells, cerebellar, hippocampal, etc.). The inhibitory response occurs through presynaptic axon synapses are that take place generally between GABAergic interneurons and afferent primary excitatory coming on spinal motor neurons. The GABAA receptor is a pentamer consisting of at least two different polypeptide subunits; been identified so far are six isoforms of subunit (to α1-α6), three β (β1-β3), three γ (γ 1-γ3), two ρ (ρl - ρ2) and a σ. The GABAA receptor is present in different combinations but with combinations rather limited, the main one is formed by two subunits of α, two subunits of β and one of γ. This type of receptor has a synaptic localization and is involved in mediating a current inhibitory type “Phasic”; results to have a low affinity for the GABA, a rapid desensitization, reduced sensitivity to neurosteroids (allopregnanolone) and high sensitivity to the benzodiazepines. In recent years, in addition to currents mediated by synaptic receptors, an activity was characterized GABAergic inhibitory defined "Tonic”, mediated through extra synaptic receptors. These receptors are characterized, in the granule cells of the hippocampus around dentate gyrus, of subunits σ and α4,

while in the granule cells of the cerebellum, of subunits α and α6. These receptors are also characterized by a high affinity for the GABA and by a reduced rate of inactivation, in agreement with the fact that they must be brought into activity by a small amount of GABA which diffuses away from the synaptic cleft (spillover); the one is high sensitivity to neurosteroids and other is not sensitive to benzodizepine. This tonic current has an important role to control neuronal excitability.

Some recent experimental evidences have suggested a mechanism of action of ethanol directed towards the positive modulation of the GABAergic system, or according to which they could be explained some of its effects CNS depressants (Morrow *et al.*, 2001). These effects could be mediated by the stimulation by ethanol, steroidogenesis, with the resulting formation of steroids which act actively, at the central level, by modulating the function of GABAA receptors, like other active substances at the level of the GABAergic neurotransmitter system. Such as barbiturates, benzodiazepines, neurosteroids like allopregnanolone, ethanol also has an inhibiting effect of the central nervous system through its direct and indirect action on the GABAegic system (Grobin *et al.*, 1998). The result of this action causes behavioural disinhibition, anxiolytic, sedative hypnotics, anticonvulsants and muscle relaxants such as GABA agonists.

Glutamatergic Transmission

Glutamate is the most common excitatory neurotransmitter in the CNS. It has been well established that many functions of the brain (the perception ordered sensations and pain, learning, memory, control of motor function). Alteration of glutamatergic then seems to underlie diseases such as seizures, neuronal death ischemic or hypoglycemic, degenerative diseases, cerebral aging.

The glutamate is distributed fairly evenly in all regions of the brain and is present in neuronal glial cells. Since the blood-brain barrier (BBB) is in fact almost impermeable to glutamate, it must be synthesized in the nervous system. The most important for the new synthesis of glutamate with neurotransmitter function is formed by deamination of glutamine. This can diffuse from the blood into the central nervous system (but also the glia to neurons) which can be metabolized by the glutaminase, an enzyme found in excitatory neurons and capable of turning it into glutamate. In nerve endings glutamate is concentrated in synaptic vesicles. The main mechanisms for switching off the signal of the glutamatergic synapses are represented by the efficient transport systems both of the neurons that glia, which transfer glutamate from outside to inside the cell.

For glutamate, there are three classes of receptors-channel; it is multimeric receptors consist of 4 or 5 subunits that participate to form a channel open to interaction with the transmembrane ion glutamate. They differ in terms of kinetics of activation/inactivation, kinetics desensitization, permeability and ion conductance. They are classified into three categories according to the agonist selective for the AMPA receptors (alpha-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid), kainate receptors and receptors for the NMDA (N-methyl-D-aspartate). The AMPA receptors have kinetics of activation/inactivation and desensitization very fast (millisecond), they are permeable to Na^+ and less to Ca^{2+}; are mainly located in the postsynaptic membrane and are responsible for the response excitatory (depolarizing) rapid typical of

glutamatergic synapses function of specific receptors for the kinate (not activated from AMPA) is less clear; they are disseminated throughout the CNS but less abundant AMPA receptor.

The NMDA active a particular type of receptor-channel equipped with much more kinetics electrophysiological lens and high ion permeability Ca^{2+}. The infusion of Ca^{2+} through this channel is an important consequence of the activation of glutamatergic synapses, it is only if the postsynaptic membrane is depolarized enough (through activation of AMPA receptors) in order to remove the block of Mg^{2+} ion exercises at the level of ion channel receptor. The influence of Ca^{2+} mediated by the NMDA receptor has important biological effects on neuron, these range from the effects of trophic type, effects of regulatory type of synaptic transmission and finally a kind of toxic effects that may result in neuronal death. Glutamate is the main neurotransmitter involved in the control of synaptic plasticity of the brain or in the synapses that exhibit phenomena of LTP (long-term potentiation) and LTD (long-term depression). These two forms of neuronal plasticity withhold base day important cognitive processes and learning day. In basal conditions glutamate is released from the presynaptic terminal into the synaptic space, and will go to interact with their AMPA receptors in postsynaptic level, generating an excitatory postsynaptic potential transient. When instead of normal stimulations, the most intense (such as high frequency stimulation) are delivered, we will have a greater input of Ca^{2+} ions and will be released proportionally a greater amount of glutamate that will go to interact with NMDA receptors. Which are sensitive to a more intense depolarization. This depolarisation has the role of eliminating the Mg^{2+} ion which blocks the cation channel of the NMDA receptors making them able to conduct Ca^{2+}. The entrance of Ca^{2+} in the cell results in the activation of a cascade of Ca^{2+} dependent reactions, as activation of the kinase, calmodulin, PKC that amplify the signal. As a result we will have the CAMkinase type 2 phosphorylates. AMPA receptors on the membrane making them more sensitive and also increases the number of AMPA receptors at post synaptic level. The Ca^{2+} also activates nitric oxide synthase (NOS). An enzyme capable of catalyzing the production of nitric oxide (NO), an important

messenger with retroactive action, which could regulate the release of presynaptic glutamate. These factors will lead to having as a final result an excitatory post-synaptic potential (EPSP). The persistence of LTP for hours and days requires gene transcription (nuclear) and protein synthesis (at dendritic). Kinase activated by Ca^{2+} gives signal to the nucleus, to activate gene transcription of important factors, such as CREB (cAMP response element binding protein), which control the synthesis of proteins essential to the maintenance of LTP, fundamental for changes in the shape and increase in the size of the synapses, as well as for the generation of new dendritic spines and thus increase the contact between cells (this is the basis of training and memory consolidation). While with the high frequency stimulation are obtained concentrations of Ca^{2+} higher than 5mM, with low frequency stimulation (500stimuli, 1Hz), the concentrations Ca^{2+} should not the 5mM. With the LTD fact they observe the opposite phenomena to those seen previously for the LTP; with such low concentrations day Ca^{2+} which activated phosphatase and there is a reduction of the sensitivity of AMPA receptors to glutamate, reducing the release over NO; phenomena which lead to a reduction of the response which persists however in time.

These phenomena of plasticity assume an enormous importance at the level of central neurons, particularly in neurons of the hippocampal system. An event completely physiological short duration is thus able to cause a lasting synaptic modification, after the high frequency stimulation, the target neuron reinforced by LTP produces excitatory postsynaptic potential (EPSP) wider for a long time.

Anatomy of the hippocampal formation

The hippocampus, a structure located under the convoluted lateral ventricle, includes the hippocampus itself, the dentate gyrus and the subiculum. In humans, the regions are located in appearance the parahippocampal gyrus of the hippocampus and the entorhinal cortex (Brodmann area 28) the appearance of the basolateral parahippocampal gyrus. The hippocampus proper (also called Horn of Ammon) was divided by Lorente de Noh (1934) in subregions CA1, CA2, CA3 and CA4. These sub regions have been distinguished more precisely in rats by Blackstad (1956) is a vast region of the CA1 pyramidal neurons dispersed small (in humans) and adjacent to the subiculum. In the rat CA1 pyramidal neurons they are strongly thickened. The CA2 in humans is a distinct region between the CA1 and CA3 is made up of large pyramidal neurons ovoid strongly thickened. In rats CA2 is smaller and less distinct. The CA3 is localized in the curve of the hippocampus, where this fits in the dentate gyrus CA3 pyramidal neurons which are similar to those of the region CA2 but less thickened. They are also distinguished by the presence of a strong innervation of mossy fibers from the granule cells of the dentate gyrus. The region inside the concavity was called CA4 by Lorente de Noh, but today commonly called hilum.

The Horn of Ammon is divided into six layers. The first is called alveus, where there are the axons of pyramidal neurons and inter-neurons of the underlying layer pyramid GABAergic neurons mediating an injunction. It follows the pyramidal layer with the bodies of pyramidal glutamatergic neurons. Then we observe the layer radiatum and lacunosum, where we find other inhibitory interneurons and dendrites of pyramidal neurons. Finally we have the molecular layer. The dentate gyrus is rich in granule neurons much smaller than observed in pyramidal superstructure pathways afferent fibers coming from the entorhinal hippocampus include the parahippocampal gyrus, fibers cohnergiche septal area and the substance unnamed, dopaminergic fibers from segmental ventral noradrenergic fibers, from the locus coeruleus, and serotonergic fibers from the raphe nuclei. The efferent pathways enter the Papez circuit, which includes the

hippocampus, the fornix, mammillary body, the anterior nuclei of the thalamus, the cingulate gyrus and the convolution parahippocampal fibers. Association connect the cingulate gyrus and parahippocampal to large areas of the neocortex. The major intrinsic hippocampal synaptic pathways can be divided into circuits lamellar, longitudinal and local increased via excitatory is trisinaptic. It comes from the entorhinal cortex across the street piercing that forms synapses with granule neurons of the dentate gyrus. The axons of these neurons, forms synapses with dendrytes of CA3 pyramidal neurons of the area through the path of mossy fibers. Fibre side of CA3 neurons are forms synapses with GABAergic interneurons both pyramidal neurons of the CA1 through the Schaffer collateral fibers. In turn, the CA1 pyramidal neurons projecting their side axon back to the entorhinal cortex. The excitatory synapses are predominantly glutamatergic while all inhibitory circuits, which have a predominant action control excitability of excitatory neurons, are mainly GABAergic. Within the sub-region of the hippocampus and dentate gyrus, therefore, the principal cells and interneurons form synaptic local circuit that influence hippocampal function in important ways. The granule cells send long side to many interneurons, particularly the mossy fibers, the hilum which in turn create contacts with other hilum interneurons and granule cells (Hjort-Simonsen and Laurber, 1977 Scharfman *et al.*, 1990). CA3 pyramidal cells have extended local collateralizzazioni axonal and excite the neighboring cells CA3 and inhibitory interneurons (Miles and Wong, 1986; Ishizuka *et al.*, 1990; Miles, 1990). Interneurons in CA3 inhibit pyramidal cells and other interneurons (Miles and Wong, 1984), inside the area CAl, pyramidal neurons send axonal collateral eccitatone to several classes of interneurons inhibit the pyramidal cells and other interneurons (Knowles and Schwartzkroin 1981 a,b ; Lecaille *et al.*, 1987). It is therefore evident that the hippocampal excitability is mediated by an equilibrium between the two systems, glutamatergic and GABAergic. In order to then determine the degree of neuronal excitability is necessary to analyze the functionality glutamatergic that undergoes changes mediated by interaction with the GABAergic system is therefore evident that stressful events, as we have seen, or

interaction with drugs that affect the GABAergic system, induce a modification of glutamatergic function and consequently an overall variation of neuronal excitability. Endogenous like neuroactive steroids (products peripherally and centrally active) or neurosteroids (produced centrally) are especially active as positive modulators of GABA; so stressful situations that alter their physiological homeostasis and therefore their actions on the GABAergic system may influence the activity of brain areas such as the hippocampus.

Amygdala

Amygdala is known in neural circuitry for emotion (Gallagher and Chiba, 1999). It is an almond shaped structure deep within the temporal lobe. It is a complex structure consisting of 20 nuclei which are further divided into extensive internuclear and intranuclear connections (Sah *et al.*, 2003). These are mainly divided into a deeper basolateral group (BLA), cortical group, and a centromedial group (CeA). Primary sensory input zone of amygdale is BLA and the primary output structure is CeA. (Marek *et al.*, 2013, Sah *et al.*, 2003, Pape and pare, 2010). Glutamatergic neurons are the principal neurons of BLA. While CeA contains GABAergic neurons. (Marek *et al*., 2013).

Amygdala plays a key role in controlling fear and anxiety. This neural circuitry has been studied in details by several group of research (Blanchard DC and Blanchard RJ, 1972; Bechara A. *et al.*1995; Labar KS *et. al*. 1995). Studies shown that the amygdala, including the basolateral complex and the CeA, plays key roles in the acquisition and expression of fear-related behaviors. Fear conditioning is associated with uncontrolled stimulus such as mild foot shock which are typically used as unconditioned stimuli. Blanchard DC and Blanchard RC have shown that, in rats when lesions are given to the amygdala disrupt Pavlovian fear conditioning (Blanchard DC and Blanchard RJ, 1972). Bechara A. *et al.*(1995) and Labar KS *et. al*.(1995) shown that lesions in amygdala disrupt fear conditioning in non-human primates and humans.

AIM OF STUDY

Stress produced by repeated maternal separation (RMS) during the first two weeks of postnatal life plays very important role in the neurobiology of addiction developed in adulthood. Several evidence report that animals which are exposed to RMS stress increases voluntary ethanol consumption (Roman and Nylander, 2005). Some literature reported that estrogen plays not only a role as a modulator of stress and anxiety (Morgan and Pfaff 2001, 2002) but are also able to induce synaptogenesis in the hippocampus (Kretz *et al.*, 2004). We made some experiments to evaluate the ability of estrogen to antagonize the effects induced by the RMS on voluntary ethanol consumption and alterations in function of GABAergic and GLUergic systems. Based on literature review, we decided to evaluate the ability of estrogen to antagonize the effect induced by the RMS on voluntary ethanol consumption in adulthood and function of hippocampal excitatory and inhibitory synapses.

We have decided to use behavioral animal model of voluntary ethanol consumption and electrophysiological intracellular patch clamp technique, to record intracellular currents as well as extracellular field potential technique, to record the synaptic plasticity in terms of long term potentiation (LTP) and long term depression (LTD) in the CA1 area of hippocampus.

We have selected the hippocampus and central nucleus of amygdala to study the changes in function of GABAergic and GLUergic system due to effect of repeated maternal stress and effect of β-ethinyl estradiol. We have selected granule neurons of dentate gyrus and pyramidal neurons of CA1 as well as central nucleus of Amygdala to record GABAergic inhibitory and GLUergic excitatory currents.

MATERIALS AND METHODS

Animals

Mice of the C57BL/6J strain are used for the experiments. The animals were obtained from Charles River, Italy. After breeding them in our animal facility, the new born animals, both the males and females, were used for the experiments. They were housed in a controlled temperature (22°C) and humidity (65%). Also, 12h dark/light cycle was maintained. Animals were provided to ad libitum access to food and water. Animals were treated according to the Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Experimental protocol is approved by animal ethics committee of University of Cagliari.

Protocol of repeated maternal separation

C57BL/6J mice pups were divided into two groups, group housed and those which were going to be subjected to repeated maternal separation. Group housed animals were kept with their mother at all time, this was the control group of animals. Whereas animals which were subjected to repeated maternal separation protocol, were separated from their mothers for 360min (from10am to 16pm), starting from post natal day 3 (PND3) until post natal day 17 (PND17). The pups were separated from their mothers, placed in a cage, and transferred into a different room. The temperature of the room was maintained at 32°C. In group housed animals, the positions of the pups were changed at 10am and 16pm. But the pups were always kept with their mother.

After the weaning (PND21), animals were kept in a group of 5 animals per group till post-natal day 60 (PND60).

Protocol of voluntary ethanol consumption

The adult mice (PND60) that have previously been exposed to RMS and the control mice (which have not undergone the RMS, GH) each were housed in individual cages to quantify the voluntary consumption of ethanol by the protocol of free choice (Free-choice) between ethanol and water. The entire protocol provides for a period of six weeks, during which the animals had the opportunity to choose between two bottles, one containing water and the other a solution of ethanol. Free choice was given for two hours a day. During the first 10days of treatment, the mice were offered a solution containing ethanol mixed with sucrose. The sucrose-fading procedure was employed to further stabilize their daily fluid intake. The solution contained different concentrations of both ethanol and sucrose; on day 1-2, EtOH 10% and sucrose 5%; on 3-4 EtOH 12% and sucrose 5%; on 5-6, EtOH 15% and sucrose 5%; on days 7-8 EtOH 15% and sucrose 2%; on days 9-10 EtOH 15% and sucrose 1%. And finally for the subsequent weeks of treatment a solution containing only 15% EtOH. The different EtOH and sucrose solutions were prepared as v/v and w/v solutions, respectively. At the end of the 2h access period, EtOH and water bottles were removed and the one standard water bottle was returned to the home cage. The EtOH solutions were presented at room temperature. EtOH (2h) and water (2h) intake was measured daily by weighing the bottles. The difference with the initial weight was calculated in order to establish the amount of fluids drank during the 2h period. Dependent variables recorded and analyzed include EtOH intake (g and g/Kg), water intake (g), and total fluid intake (EtOH as well as water intake). EtOH preference ratio was calculated as the volume of EtOH, divided by the total volume of liquid (EtOH as well as water) consumed. At the end of the 6-weeks period, the last 2h session of the two-bottle choice.

The animals which are subjected to this protocol, are kept in reverted dark/light cycle.

Injection of β Ethinyl-estradiol

In a separate group of male mouse pups, the second day after birth (PND2), a subcutaneous injection of β Etinyl-estradiol was given. β Etinyl-estradiol was prepared by

dissolving it in sesame oil. Sesame oil is used to get final concentration of 25µg/mL. In control animals, only vehicle, which is sesame oil, in injected by subcutaneous route.

After injecting β Etinyl-estradiol, animals were treated as per the experimental groups of group housed animals, or animals treated with repeated maternal separation protocol.

Preparation of brain slices

The C57BL/6J mice were deeply anesthetized by inhalation of vapours of chloroform and decapitated. Brain was rapidly removed and dipped in a ice-cold cutting solution containing in mM, 220 sucrose, 2 KCl, 0.2 $CaCl_2$, 6 $MgSO_4$, 26 $NaHCO_3$, 1.3 NaH_2PO_4, and 10 D-glucose. After one min, prefrontal cortex and cerebellum was cut from brain. Then it was pasted on a metal disk with the help of glue containing cyano acrylate. Using a Leica VT1200S vibratome, slices were prepared in the coronal section (thickness from 250-300µm) containing the region of interest (dorsal hippocampus, central amygdale). Slices were then transferred immediately to a nylon net submerged in normal ACSF, for at least 40min at 35°C controlled temperature. A standard artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 D-glucose (pH 7.4, was prepared and set by aeration with mixture of 95% O_2 / 5% CO_2). Then, following at least 1h of incubation at room temperature, hemi-slices were transferred to a recording chamber, submerged in normal ACSF with a constant flow of ~2ml/min. For all recordings, the temperature of the bath was maintained at 33°C.

Patch clamp recordings in whole-cell configuration

Electrophysiological patch clamp recordings in the whole-cell configuration were performed at the level of granule neurons, in the dentate gyrus (DGGCs), and pyramidal neurons of the hippocampus CA1 field, with recording electrodes that were prepared from borosilicate capillaries with internal filament and an external diameter of 1.5 using a Flaming Brown Micropipette puller (Molecular Devices, Novato, CA). The resistance of the electrodes varied from 2.5 to 4.5 when such

electrodes were filled with an internal solution containing CsCl 140mM, 2mM $MgCl_2$, 1mM $CaCl_2$, 10mM EGTA, 10mM HEPES, ATP-Na_2, pH 7.3 with CsOH 5N. Currents through the patch clamp amplifier, were filtered at 2kHz, digitized at 5.5kHz, and analyzed with the software pClamp 10.2 (Axon Instruments). To record Cl^- currents evoked by GABA, kynurenic acid (1mM) was added to the solution of recording ACSF to block receptor-mediated glutamatergic currents. For all experiments, cells were clamped at a voltage of -65mV. Spontaneous inhibitory postsynaptic currents (sIPSC) were recorded using the software pClamp 9.2; to analyze current amplitude, decay time, rise time, and frequency the software Minianalysis 6.0 was used. In the experiments for the analysis of the tonic current of the DGGCs, THIP (partial agonist of GABAA receptors) was perfused for 6min, in order to positively modulate the GABAergic tonic current. After perfusion of THIP, bicuculline (20μM), a GABA-A receptor antagonist, was applied. Using the same software for recording and analysis we have recorded spontaneous excitatory postsynaptic currents in the presence of GABA antagonist bicuculline (20μM).

Extracellular electrophysiological recording for induction of LTP and LTD

Synaptic plasticity in terms of LTP and LTD was recorded through a binocular microscope at low magnification (approximately 20-50x). Extracellular recording electrodes were made from borosilicate capillaries with a filament and an outer diameter of 1.5μm (Sutter Instruments, Novato, CA, USA) which were filled with 3M NaCl. For stimulation of afferent fibers, a concentric bipolar electrode was used. Slices containing the hippocampal formation were placed and Schaffer collateral pathway was stimulated. Responses were triggered digitally every 20s using an interval generator (Master 8). To quantify the response (field excitatory postsynaptic potential, fEPSP), its slope value was considered. First, the input-output (I-O) curve was recorded. For the I-O curves, stimulation current intensity ranged from 0.0 to 1.0mA with steps of 0.1mA. fEPSPs were amplified by an Axoclamp 2B amplifier (Axon Instruments, Union City, CA, USA) digitized and analyzed

using Clampfit 8.02 software (Axon Instruments). On the basis of the I-O curve, the current intensity able to evoke a half-maximal response was selected. To record long term potentiation (LTP), a high frequency stimulation (HFS) consisting of 100 stimulations at 250Hz was delivered and the recording was continued for 60min with stimulations of fEPSP every 20s, to determine the effect of HFS. To record long term depression (LTD), a low frequency stimulation (LFS) consisting of 900 stimulations of 1Hz was delivered and the recording was continued for 60min with stimulations of fEPSP every 20s, to determine the effect of LFS. The purpose of the high stimulation (HFS) and low (LFS) frequency is to increase, with a different intensity, the release of glutamate and induce the consequent activation of postsynaptic AMPA receptors and NMDA. Admission ion Ca^{2+} through NMDA receptors and through the opening of voltage-gated channels of Ca^{2+} (that are activated during intense depolarization) can induce long-term changes of synaptic activity.

The bipolar stimulating electrode, activating the presynaptic terminals of the Shaffer collaterals, induces the so called presynaptic volley. This is caused by an inward cationic currents, associated to the action potentials, in the presynaptic fibers. Secondly, there is fEPSP, which is caused by the synchronized depolarization of the population of CA1 pyramidal cells that are located in the vicinity recording electrode. A depolarization (i.e. a suprathreshold potential change that leads to the onset of an action potential) is measured as a change in a positive way from the resting potential of the cell (usually negative). For the short time of a depolarization (few ms), the extracellular fluid is found to have a deficiency of positive charges, the equivalent of an excess of negative charges. In this way, when a depolarization is induced, an electrode placed outside of a neuronal population (recording electrode) will measure a change, with negative development, which allows to observe our response, given by a negative deflection. Several kinetic parameters of fEPSP were analyzed, but slope is used for the evaluating the response.

RESULTS

Effect of RMS on voluntary ethanol consumption and preference in C57BL/6J adult mice: Difference between male, female, and male treated with β-ethinyl estradiol.

As illustrated in Fig. 1, male mice subjected to RMS for 15 days consumed significantly more amount of ethanol (3.46±0.13g/Kg), as compared to control animals (2.42±0.13g/Kg) when tested on adult life. When females were subjected to RMS, there was no significant difference in ethanol (3.20±0.21g/Kg) consumption as compared to group housed animals (3.81±0.36g/Kg). Whereas males treated with β-ethinyl estradiol and subjected to RMS for 15 days, consumed significantly lower amount of ethanol (5.41±0.26g/Kg) as compared to control animals (6.87±0.39 g/Kg) suggesting a pattern similar to what observed in females.

As illustrated in Fig. 1, the results obtained from preference (% of ethanol vs total fluid) suggest that, the GH C57BL/6J male mice preferred significantly more amount of ethanol (61.53±2.23%) as compared to water (38.47±2.23%). Also, male animals subjected to RMS for 15 days preferred significantly more amount of ethanol (71.33±2.42%) as compared to water (28.67±2.42%) but this preference is more pronounced than what observed in GH. The group housed females preferred significantly more amount of ethanol (69.06±1.71%) as compared to water (30.94±1.71%) but this effect is not more pronounced in RMS animals as seen in male mice. The group housed male animals treated with β-ethinyl estradiol showed significantly greater preference to ethanol (65.26±2.08%) as compared to water (34.74±2.08%). Whereas, the male animals treated with β-ethinyl estradiol and subjected to 15 days of RMS shown no difference in ethanol (52.00±1.86%) and water (48.00±1.86%) preference.

The time-dependent increase of GABAergic sIPSC frequency and tonic currents in DGGCs during the first 17 days of life is enhanced in male mice exposed to RMS with respect to group housed animals but not in female or male treated with β-EB.

We used patch-clamp recording in the whole-cell configuration to record spontaneous postsynaptic inhibitory currents (sIPSCs) mediated by GABA-A receptors, at the level of dentate gyrus granular cells, at various time intervals relative to the postnatal period and RMS exposure. The membrane potential of these cells was clamped to -65mV and the sIPSCs mediated by the GABA-A receptors were recorded in the presence of kynurenic acid, a non-selective ionotropic glutamate receptor antagonist, in the ACSF solution.

As illustrated in Fig.2 (A and B), the frequency of GABAergic sIPSCs, recorded dentate gyrus neurons, is significantly increased in repeated maternal separated animals compared to controls; this is evident from the seventh to the fifteenth day of RMS. In adult animals (PND 60) this difference is no longer noticeable. This suggests that changes in GABAergic system during the RMS may contribute to the behavioural differences observed in adulthood and it's interesting to note that, such variations in the frequency of sIPSCs is not apparent in female mice (Fig.2C).

In the same granule cells of the dentate gyrus of adult animals (PND 60) undergoing RMS, we studied the different kinetic parameters of sIPSC and recorded GABA-A mediated extrasynaptic tonic currents.

Effect of RMS on GABAergic transmission on dentate gyrus.

As illustrated in Fig.3, we studied alterations induced by RMS (RMS) on GABAergic transmission. We have used males, females, as well as males treated with β-ethinyl estradiol. We measured the frequency of sIPSCs on 2^{nd}, 7^{th}, 15^{th}, and 60^{th} day. We can see from Fig.3(A-C) that the alterations induced by RMS on GABAergic transmission (frequency of spontaneous currents sIPSCs) evaluated in the dentate gyrus of adult mice is not found in females exposed to RMS, as

well as in male animals exposed to the RMS and treated a single injection of β-ethinyl estradiol. As we can see in Fig.3(A), the RMS is associated with an increased release of presynaptic GABA at the level of inhibitory synapses in the granule neurons in the dentate gyrus that is highlighted during the days of separation but not in adult male mice. In females, we did not observe any significant effect associated with RMS both during exposure to this protocol and in adulthood (Fig.3(C)). The treatment of male mice with β-ethinyl estradiol antagonizes the increase in GABA release during the days of separation, when compared to that observed in animals exposed to the RMS and treated only with vehicle (sesame oil).

As illustrated in Fig.4, these are the representative traces of GABAergic tonic currents obtained from both the control animals as well as repeated maternal separated animals. We studied the tonic current with the help of patch-clamp technique in single granule cell of dentate gyrus. We have studied in various time after birth, 2^{nd}, 7^{th}, 15^{th}, and 60^{th} day. As we can see, in both control and maternal separated animals shows the effect of perfusion of GABAergic agonist, THIP in the hippocampal slice.

Fig.5 represents the scatter plots of the modulatory effects of THIP on tonic currents recorded in granule neurons of the dentate gyrus from the control mice and animals exposed to RMS. We have studied it in 2^{nd}, 7^{th}, 15^{th}, and 60^{th}. The modulatory effect of THIP on tonic currents is greater in animals exposed to RMS as compared to group housed animals only at 60^{th} day.

Effect of RMS on GABAergic and GLUergic transmission on dentate gyrus.

Fig.6 illustrates the effect of RMS on sIPSCs recorded from dentate gyrus cells. We found no significant difference between the sIPSC amplitude, rise time, decay time, and frequency in maternal separated and group housed animals.

As illustrated in Fig.7, there is a significant effect of THIP (3μM), a GABAergic agonist, on tonic currents. Noise variance (% vs baseline) was higher in repeated maternal separated animals

compared to controls. At the same time, there was no significant effect of bicuculline (20μM), a GABAergic antagonist, in group housed and repeated maternal separated animals.

Also, there was a significant increase in the effect of THIP (3μM) on tonic current holding current shift (shift vs baseline), in RMS animals as compared to GH animals. Similar effect was seen when we perfused bicuculline (20μM). There was significant increase in the effect of bicuculline on tonic current holding current shift (shift vs baseline),in RMS animals as compared to GH animals.

This results suggests, there is an increase in the functionality of the extrasynaptic GABA-A receptors which mediate tonic currents.

As we can see from Fig.8, we have studied effect of RMS on the kinetic properties of basal glutamatergic sEPSCs recorded in dentate gyrus granule cells. There is significant increase sEPSC frequency recorded in RMS animals as compared to control animals. The other parameters such as amplitude, decay time, and rise time did not vary significantly between groups.

As illustrated in Fig.9, we have used 3 experimental animal groups, control mice (GH), mice exposed to the RMS and the days PND2 treated with an injection of 25μl of sesame oil (RMS+Veh) and mice exposed to the RMS but treated with β-ethinyl estradiol on PND2 (RMS+ β-ethinyl estradiol).

It shows the effect of THIP (3μM) and bicuculline (20μM) on noise variance, (% of change Vs baseline). Animals exposed to the RMS and treated with vehicle shows a significant difference as compared to the group housed when perfused with the THIP and bicuculline. The animals which are exposed to RMS and treated with β-ethinyl estradiol also shows the difference as compared to the group housed animals, when perfused with THIP and bicuculline. But, the difference is not significant.

As illustrated in Fig.10, there was a significant increase in modulatory effect of GABAergic agonist THIP (3μM) on tonic currents. This effect of THIP includes both an increase in the noise variance is the variation of the holding current (baseline).

Effect of RMS on GABAergic and GLUergic transmission on CA1.

As illustrated in Fig.11, when we recorded effect of basal GABAergic sIPSC in pyramidal neurons of CA1 hippocampal area, there was significant increase in sIPSC frequency (Hz) recorded in maternal separated animals as compared to the control animals. At the same time there is no significant difference in sIPSC amplitude, decay time, and rise time in group housed animals and RMS animals.

As illustrated in Fig.12, we have used the benzodiazepine lorazepam (1μM) in order to assess whether the changes in the function of inhibitory GABAergic synapses could also include changes in sensitivity of the action of GABA-A receptor modulators. There is a difference in the modulatory action of the benzodiazepine- lorazepam, on GABAergic currents when measured at the level of CA1 area of hippocampus (CA1), dentate gyrus granule cells (DGGCs), and central nucleus of amygdala (CeA). In fact, in the CA1 region, the positive modulatory action of lorazepam on GABAergic sIPSCs is seen in repeated separated animals compared to the group housed animals. Where in dentate dyrus granule cells, a significant effect of the positive modulatory action of lorazepam on sIPSC is seen in animals exposed to RMS than control animals. Also, in central nucleus of Amygdala, the modulatory effect of lorazepam is higher in control animals as compared to RMS animals.

As illustrated in Fig.13, we have studied effect of RMS on kinetic properties of basal glutamatergic sEPSC recorded in CA1 area of hippocampus. sIPSC recorded in maternal separated animals is lower than the group housed animals. But this difference is not significant. There is also no significant difference between rise time, decay time, and frequency.

Effect of RMS on GABAergic and GLUergic transmission on CeA.

As illustrated in Fig.14, we have recorded basal GABAergic sIPSC in the central nucleus of Amygdala, and we found a significant difference in sIPSC frequency (Hz) recorded in group housed and RMS animals. The sIPSC frequency is comparatively lower in repeated maternal separated animals relative to the group housed animals. At the same time, there is no significant change in sIPSC amplitude, rise time, and decay time.

As illustrated in Fig.15, where we have studied an effect of RMS on kinetic properties of basal sEPSC recorded pyramidal neurons of the area central nucleus amygdala. There is significant increase in sEPSC frequency in animals exposed to RMS with respect to the group housed animals. Also, we have seen that amplitude of maternal separated animals is lower than the group housed animals. But this difference is not significant. And also there is no significant difference between decay time and rise time between maternal separated animals and group housed animals.

The long-term effects of RMS (RMS) on the function of the excitatory glutamatergic and GABAergic inhibitory synapses in the hippocampus by focusing on long-term plasticity of glutamatergic synapses (LTP).

Long term potentiation (LTP) is a long lasting enhancement in the signal transmission between two neurons after repeated high frequency stimulation. We measured the activity of the glutamatergic synapse between the afferent Schaffer collaterals (which originate from CA3 pyramidal neurons) and the dendrites of CA1 pyramidal neurons. The fEPSPs were evoked by electrical stimulation of increasing intensity; these stimulations have the effect of inducing the release of glutamate which activates postsynaptic AMPA receptors which, in turn, mediate an inward current of Na^+ which generates the negative component of the fEPSP. This protocol allows to construct the I-O curves from which we can be extrapolated the value of the intensity of stimulation that evokes a half-maximal response.

After recording the I-O curve, we stabilized the response at a stimulation intensity which is equal to that producing a half-maximal response. fEPSPs were evoked every 20s. After stabilization in which a stable baseline was recorded for 10min, a HFS, consisting of 100 stimulations at 250Hz, was applied. fEPSPs are further recorded for the 60min being evoked each 20 s.

As we can see in Fig.16, there is no significant difference in the value of the stimulation intensity inducing half-maximal response between two experimental groups.

As we can see in Fig.17(A), the effect of RMS on the long term plasticity in repeated maternal separated animals and group housed animals does not show any significant difference. Fig.17(C) shows that, 1h post HFS, the percentage of LTP in group housed animals (154.1±11.5 %) is not significantly different from the value found in RMS animals (149.3±4.14%).

Vice versa, as seen in Fig.17(B), the exposure to RMS induced a greater LTD (54±8.5%) compared to group housed animals (26±4.1%).

DISCUSSION

In the present study, we have presented various experiment protocols to evaluate the effect of RMS on voluntary ethanol consumption and alteration in GABAergic and GLUergic system in hippocampus and amygdale of C57BL/6J adult mice.

The results obtained from voluntary ethanol consumption, suggest that a stress protocol such as the RMS, given in the initial weeks of life increase markedly the voluntary ethanol consumption and preference in adulthood when compared with counterpart not exposed to the protocol. On the other hand, females which are subjected to RMS shown no significant effect in ethanol intake compared with group housed counterparts even the amount of ethanol intake results higher that what observed in males. Moreover the ethanol preference is slightly less than what observed in GH females. These results suggest that, neonatal RMS has differential impact on voluntary ethanol consumption depending on gender. This observation, already described in the literature (Nylander and Roman, 2013) may leads to a crucial role played by sex hormones, with particular regards during the first stage of life. In order to evaluate whether sex hormones such as estrogen may be the basis of the insensitivity of adult females for voluntary ethanol consumption even after RMS, we injected the β-ethinyl estradiol (EB) in new born male pups to alter the normal homeostasis of estrogen cycle and sex differentiation. We thus exposed EB-treated animals to the RMS protocol and then applied the voluntary ethanol consumption after PND 60. The results obtained from this experiment suggest that, animals exposed to RMS and treated with EB consumed significantly less amount of ethanol when compared to GH animals treated with EB. In addition the ethanol preference in EB and RMS animals is completely abolished, an effect that is actually focus of studies. These results suggest that in animals treated with EB, RMS failed to induce an increase of voluntary ethanol consumption and this pattern of effects seems similar to what observed in females

Our data are consistent with previous findings that suggest that male rodents subjected to RMS consume higher amount of ethanol in adulthood (Huolt *et al.* 2001; Ploj *et al*. 2003; Roman *et al*. 2005; Roman and Nylander 2005; Cruz *et. al*. 2008). On the other hand, other reports showed that RMS has no effect on voluntary ethanol consumption in females (Roman *et. al*., 2004).

Stress in considered to play an important role in ethanol consumption but the exact mechanism is not completely understood and needs to be further evaluated (Prendergast and Little, 2007; Clark *et al*., 2008; Miczek *et al*., 2008; Pautassi *et al*., 2010; Becker *et al*., 2011, Naylander and Roman, 2013). Several researchers used RMS protocol to study the early life events that causes long-term neurobiological changes which tends to reward and addiction in later life (Roman and Naylander, 2005; Moffett *et al*., 2007; Mickzek *et al*., 2008; Becker *et. al*., 2011; Roman and Naylander, 2013). But many factors influence the effect of RMS on ethanol consumption such as Separation condition, ethanol concentration, rodent strain and animal supplier (Roman and Naylander, 2013).

In order to identify some of the neurobiological mechanisms involved in those changes at behavioral level, related to RMS, we have carried out different electrophysiological experiments in several brain areas potentially involved in the modifications observed in adults animals. We focused our attention on potential alteration of inhibitory GABAergic and excitatory GLUergic system onto hippocampus and amygdala.

We first recorded sIPSC currents mediated by GABA-A receptors activation, at the dentate gyrus level, the first and main input of the hippocampal formation. The frequency of GABAergic sIPSCs recorded in the DGGCs, is significantly increased in RMS animals as compared to GH animals. This difference is evident in initial days of RMS, while is no longer noticeable in adult animals. The increase in frequency of sIPSC suggests a parallel increase of GABA release probability. These results suggest that changes in GABAergic system during RMS may contribute to the behavioural differences observed in adult animals. Such variation in the frequency is not

apparent in female mice. Also, there is no significant difference of effect of RMS in sIPSC amplitude, rise time, decay time, and frequency recorded in the DGGCs. Interestingly in animals treated with β-EB, we have a not significant increase in sIPSC frequency in initial days of treatment as observed in females. Data related to the increased GABAergic transmission in RMS animals results very interesting and might be in line with the new findings (Biggio *et al*., 2014) in which the authors reported a protective effect of RMS on negative modifications induced by social isolation. Social isolation (SI) in weaned rats (PND21) is related to a decrease in GABAergic transmission due to both a decrease in different type of GABAAR subunits and blood/brain levels of neuractive steroids (Serra *et al*., 2007 review). Since levels of GABA transmission are increased in RMS rats at PND17 when compared with controls, it is possible to speculate saying that animals that already have high levels of GABA in the brain are also less sensitive to SI-induced decrease of those levels. This latest data may be explain the reason why RMS animals are protected by SI-induced marked effects on GABAergic system but this idea is still a focus of new studies.

We thus evaluated also the tonic component of GABAergic inhibition in the DGGCs and the possible effect of RMS in both males and females and males treated with β-EB. When we studied the effect of RMS on the potentiation of tonic current induced by THIP, we found that there is a significant increase in tonic current measured in RMS when compared to control animals. But, at the same time there is no significant difference in effect of bicuculline, suggesting that there is an increase in the functionality of the extra synaptic GABA-A receptors which mediates tonic currents.

We have also studied the effect of RMS on glutamatergic sEPSC in hippocampal formation (CA1, DGGCs) and in amygdala. In the hippocampal formation there was a change in sEPSPC frequency only on the DGGCs but not in CA1. Moreover, parameters such as amplitude, rise time, decay time (data not shown) are not involved in those changes in both subregions regions. In the central nucleus of amygdala, there is significant increase in sEPSC frequency in RMS animals as compared to control animals. This latest data may be in agreement with several reports in which it

has been reported that glutamate plays a crucial role in fear and synaptic plasticity in the amygdala complex. The increase of GLU function in this brain area may involve impairments in anxiety states observed in RMS animals.

Starting from the modification that we observed at synaptic level, we suppose that synaptic plasticity in the hippocampal circuitry may be also hampered in RMS animals. The hippocampus is a target of stress hormones (Mc Even *et al*., 1999). Researchers shown that stress affects spatial learning and memory in various experimental designs such as Morris water maze (de Quervain *et al*., 1998), hippocampal LTP and LTD (Foy *et al*., 1987; Kim *et. al*., 1996). RMS given in initial days impairs hippocampal dependant memory in the form of LTP (Sousa V.C. *et al*., 2014).

We have recorded I-O curve in CA3-CA1 excitatory synapses of hippocampus and we observed that there was not any difference in neural excitability between RMS and GH animals. We thus evaluated the synaptic plasticity forms such as the experimentally induced LTP and LTD using a high frequency and low frequency stimulating protocols respectively. Our data reported that RMS showed a slight but not significant decrease in LTP formation when HFS was applied. On the other hand RMS mice showed a greater LTD formation in response to LFS. These results suggest that only such forms of synaptic plasticity are modified by RMS and that LTP and LTD could be affected differently by this experimental protocol. These results seem consistent with recent experimental evidence that combining reduced levels of LTP and parallel increases in levels of LTD in animals subjected to chronic stress, with a state like depression observed in adult animals (Marsden, 2013).

Overall the results suggest that stress in neonatal animals exposed to RMS is associated with marked changes in the mechanism of presynaptic release of GABA and glutamate in hippocampus. These changes, in turn, taking place during the first days of neonatal life crucial for proper development of the central nervous system it might be a neurobiological factor that increases risk for increased vulnerability of adult animals to ethyl alcohol, as well as other drugs of abuse

(Gilmerand McKinney 2003; Moffett *et al*., 2007; Sanchez *et al*., 2001). In addition, the effects induced by the RMS on increase of ethanol consumption and impairment of GABAergic as well as GLUergic systems in both amygdala and hippocampal formation, were not found in female mice and in male mice which are treated with β-ethinyl estradiol, suggesting that the alteration of the physiological mechanisms underlying the sexual differentiation, caused by treatment with β-ethinyl estradiol, highlighting a potential role "protective" estrogen targeted antagonism of some of the effects of the RMS.

Taken together, the data obtained in this thesis suggest that specific stress insult during early stage of life may markedly contribute to the onset of behavioral as well as neuropsychiatric disorders occurring in adulthood. RMS-related impairments affect particularly certain neurotransmitter systems such as GABA and Glutamate that are crucial to the physiologic function of brain areas like hippocampus and amygdala. The understanding of the phenomena related to stress suffered early in life and the consequent long term modifications, is crucial to find more complex and directed pharmacological approach for the treatment of certain diseases related to stress such as alcohol dependence. Further studies on these lines are required to analyze in detail the effect of estrogen. It will be interesting to understand the possible role of estrogen in different maternal separation protocols like RMS15min vs RMS180-360 min even in different strain of animals as well as different brain areas.

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FIGURES AND TABLES

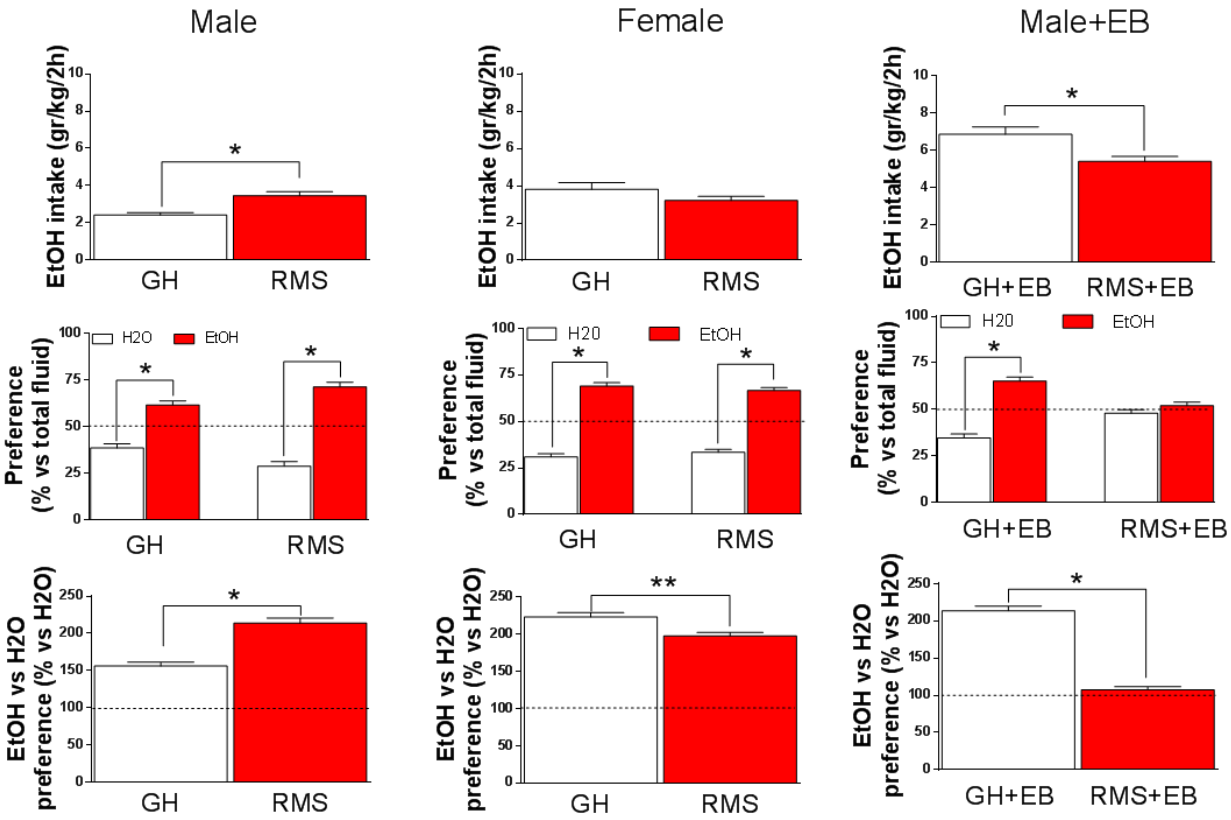

Fig.1: Effect of maternal separation on voluntary ethanol consumption in different groups of animals tested at PND60. The data are relative to male and female, group housed animals (GH) and RMS animals as well as male animals treated with β-ethinyl estradiol. *p<0.05, t-Test, BE= β-ethinyl estradiol

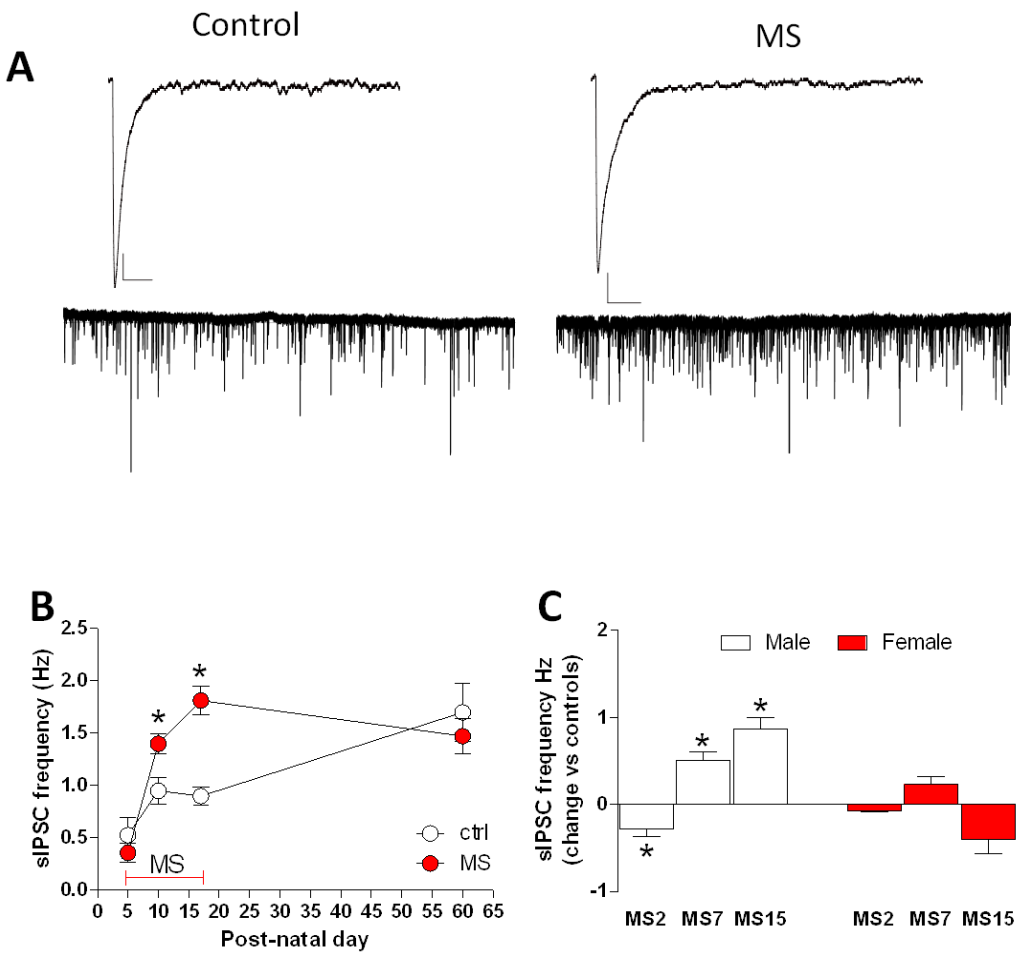

Fig.2: Effect of RMS on the kinetic properties of the basal GABAergic sIPSCs recorded at the level of granule neurons of the dentate gyrus. **A.** Traces representing sIPSCs recorded from control mice and mice subjected to repeated MS, and average currents (top). **B.** Frequency of sIPSC measured at 2^{nd}, 7^{th}, and 15^{th} days of MS. **C.** Change the frequency (Hz) of sIPSCs in animals (male and female) subjected to RMS compared to their GH controls.

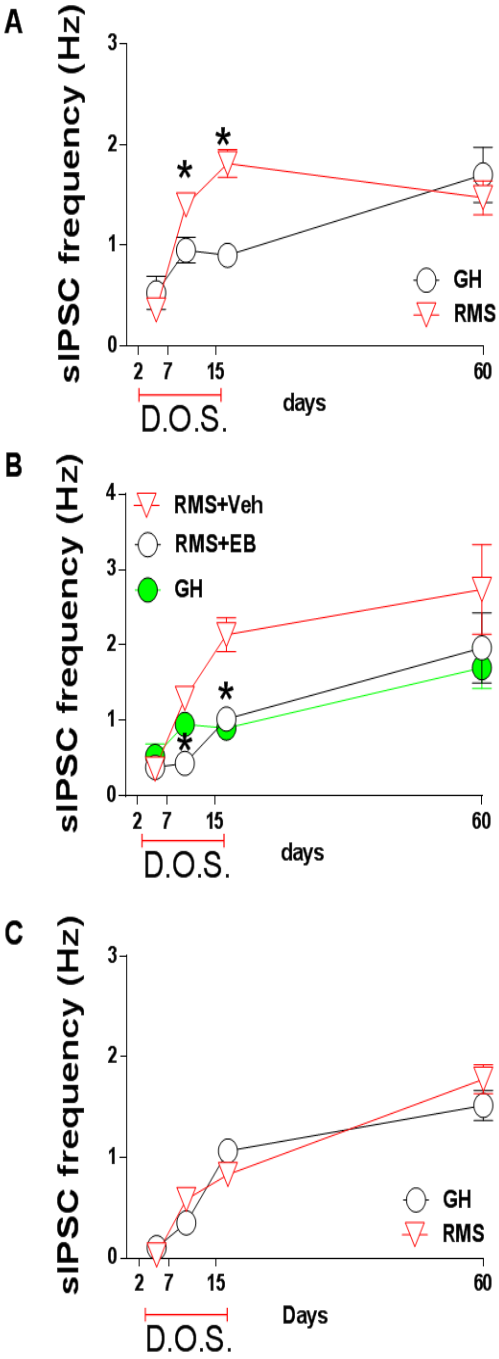

Fig.3: Effect of RMS on the properties of the basal GABAergic sIPSCs recorded at the level of granule neurons of the dentate gyrus of C57BL/6J male, female, and male treated with β-ethinyl estradiol. **A)** Frequency of sIPSC measured at 2nd, 7th, 15th, and 60th day of maternal separation in males. *p<0.05 vs. GH. **B)** Frequency of sIPSCs measured at 2nd, 7th, 15th, and 60th day of maternal separation in males treated with β-ethinyl estradiol during PND2. *p<0.05 vs. RMS+Veh **C)** Frequency of sIPSC measured at 2nd, 7th, 15th, and 60th day of maternal separation in females.

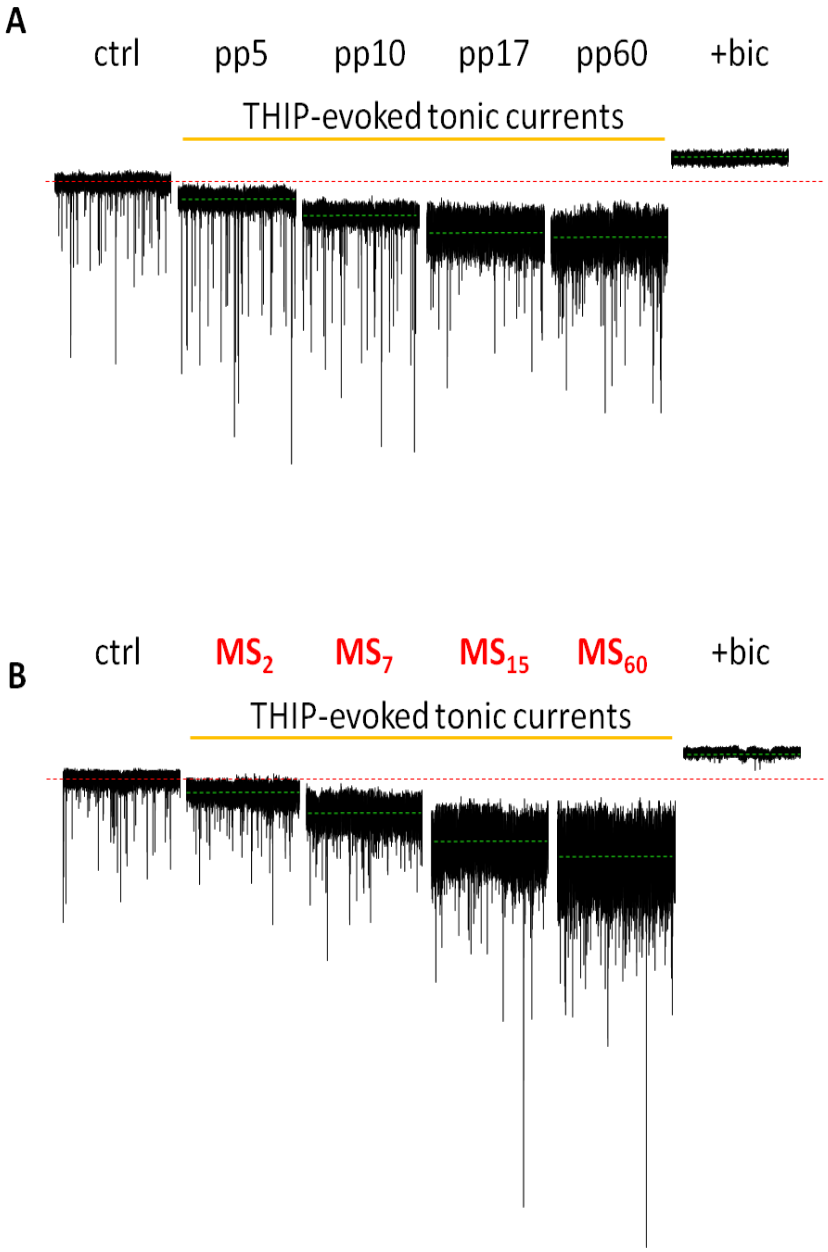

Fig.4: Representative traces of GABAergic tonic currents obtained from both the control animals as well as repeated maternal separated animals, at various times after birth.

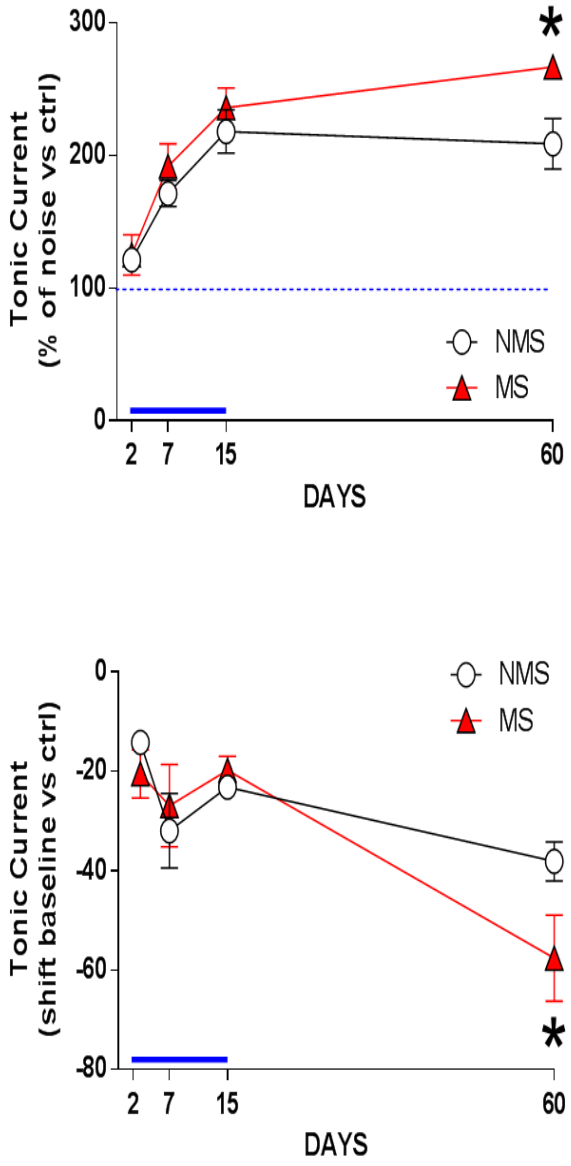

Fig.5: Scatter plot of the effects of THIP on tonic currents recorded in granule neurons of the dentate gyrus from control animals and animals exposed to RMS. The graphs show the effect of THIP on the noise variance and on the displacement of the holding current. * p <0.05 vs. GH**.**

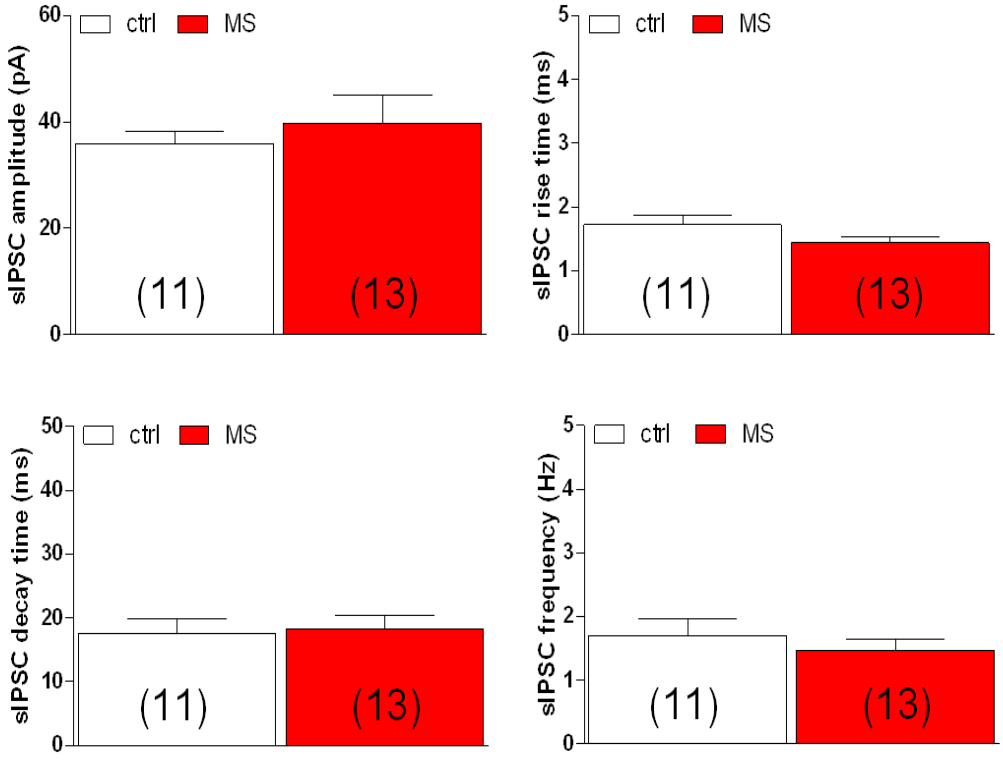

Fig.6: Effect of RMS on kinetic characteristics of basal sIPSC (amplitude, rise time, decay time, and frequency) recorded at the level of the dentate gyrus of the C57BL/6J mice.

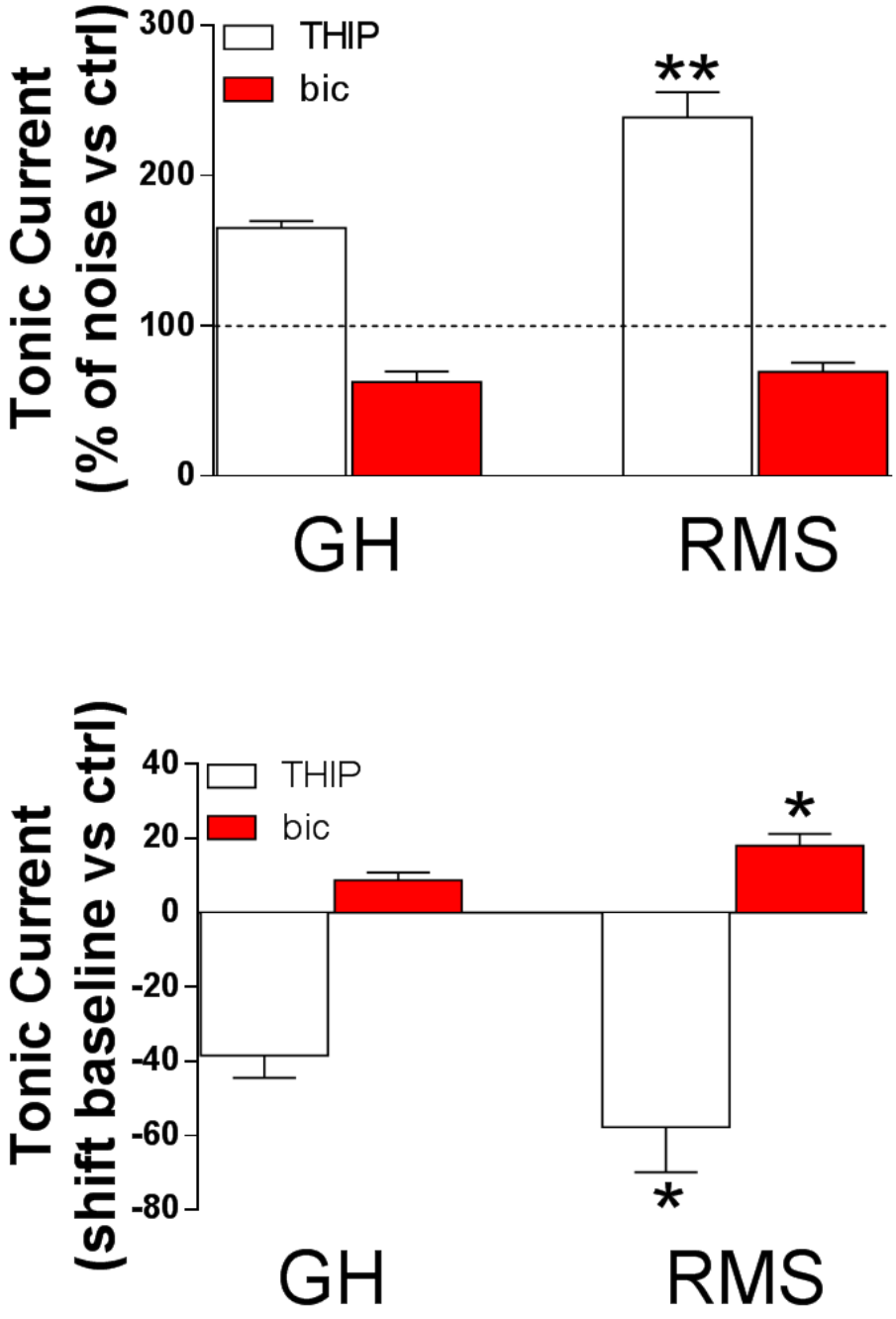

Fig.7: Effect of RMS on the potentiation of tonic current induced by THIP. The effects of THIP (3µM) and bicuculline (20µM) were measured on the noise variance (% of baseline) and the shift of the holding current (absolute values in pA). The numbers inside the bar indicate the value of "n". *p <0.05 vs. control, t-Test.

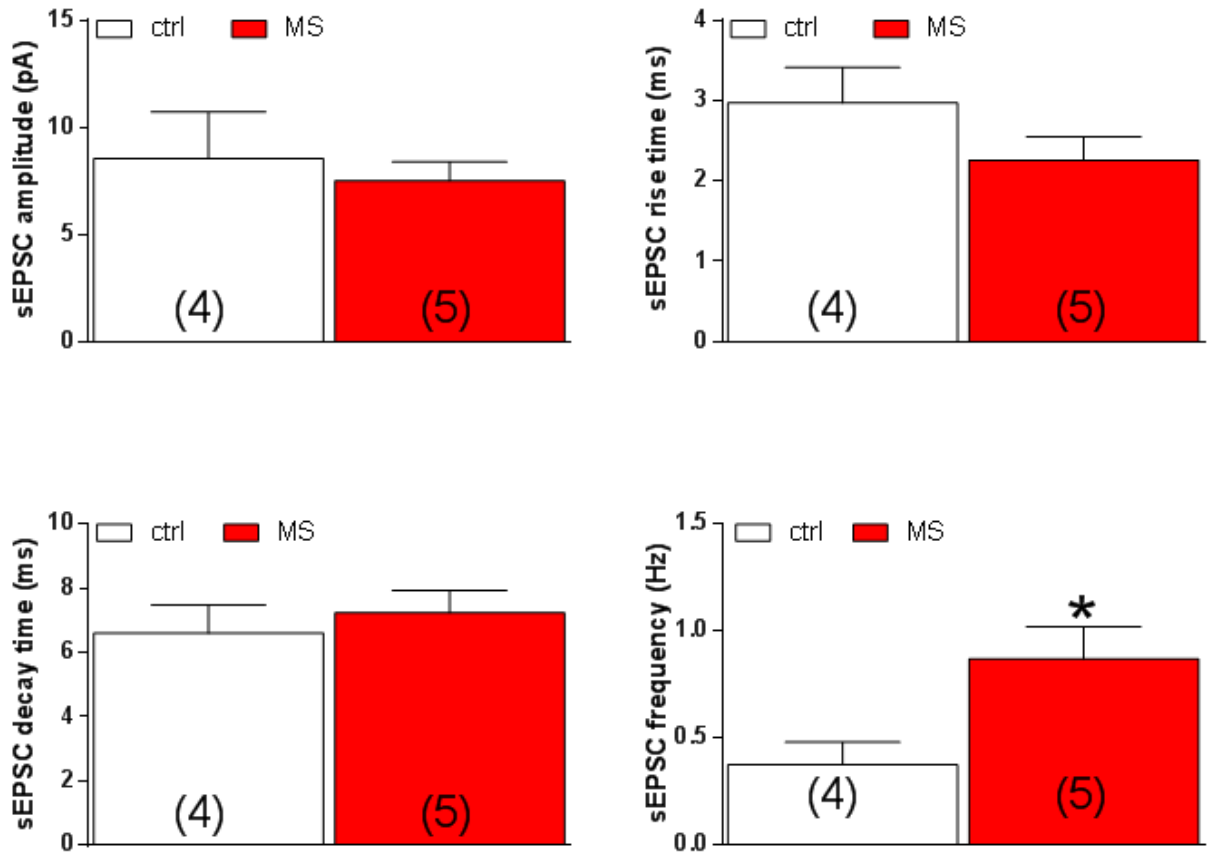

Fig.8: Effect of RMS on kinetic properties of basal glutamatergic sEPSC recorded in dentate gyrus granule cells. Values are expressed in absolute values for the different parameters. The numbers inside the bar indicated the value of "n". The numbers inside the bar indicated the value of "n". *p<0.05 vs. control, t-Test.

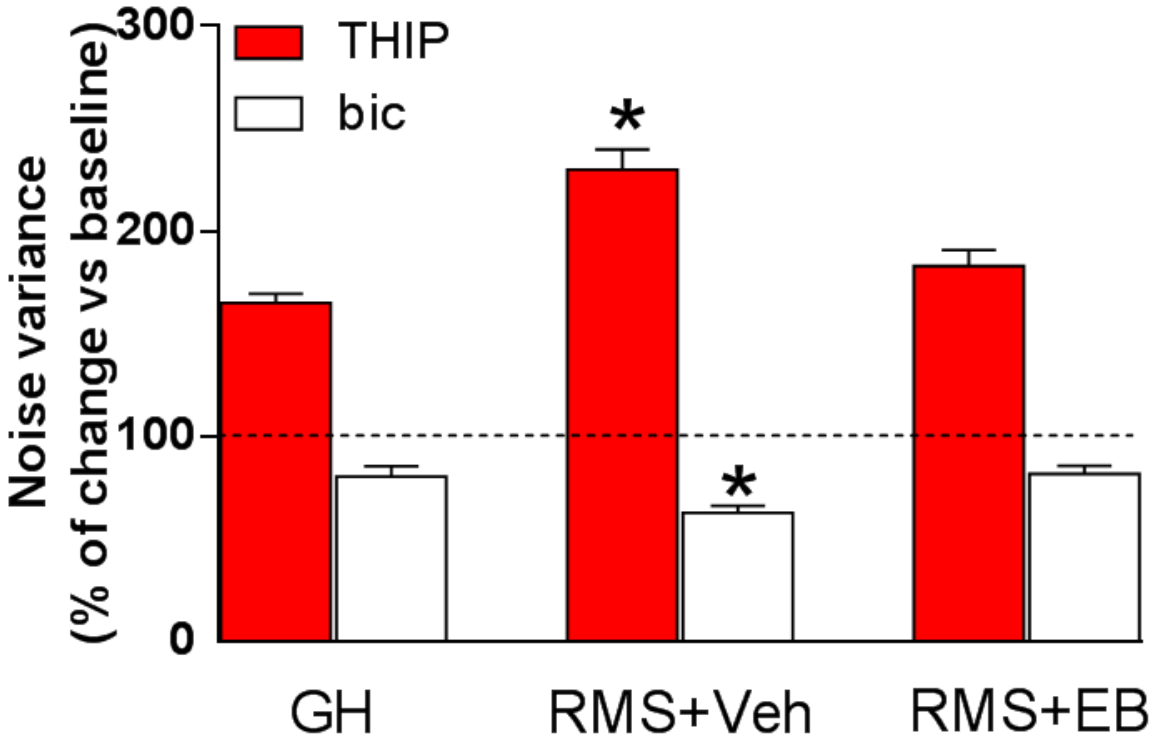

Fig.9: Effect of RMS on modulatory effect of THIP (3μM) and Bicuculline (20μM), on GABAergic tonic currents recorded in the granule cells of the dentate gyrus of the group housed animals, animals subjected to RMS and treated with vehicle, and animals treated with β-ethinyl estradiol.*$p<0.05$ vs GH.

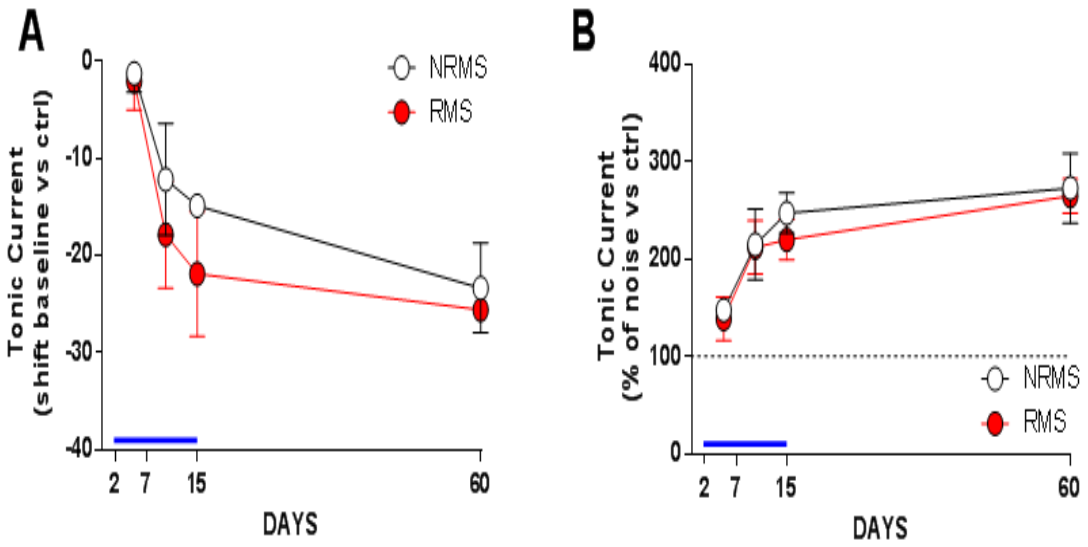

Fig.10: Effect of RMS on tonic currents measured in granule neurons of the dentate gyrus of C57BL/6J female. **A)** Modulatory effect of THIP (3µM) on the shift of the base line measured at 2^{nd}, 7^{th}, and 15^{th} day of maternal separation as well as on the 60^{th} day of life. **B)** Modulatory effect of THIP (3µM) on the variance of the noise measured 2^{nd}, 7^{th}, 15^{th}, and 60^{th} day of maternal.

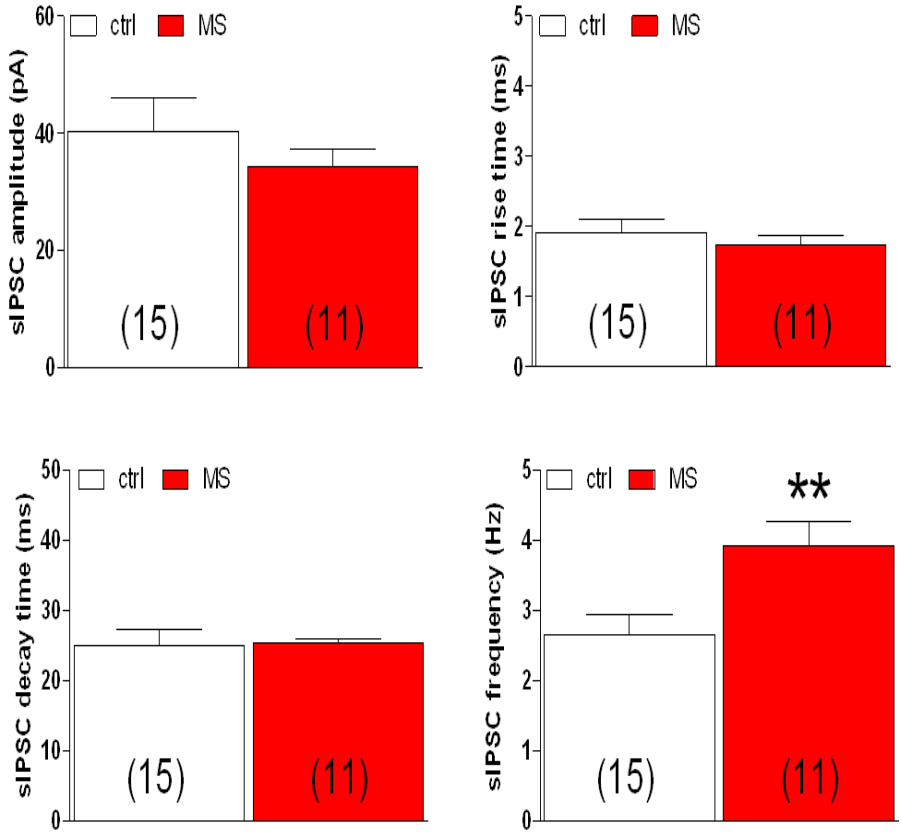

Fig.11: Effect of RMS on kinetic properties of basal GABAergic sIPSC recorded in pyramidal neurons of the hippocampal CA1 region. *Values are expressed in absolute values to different parameters. The numbers inside the bar indicate the value of "n". *p<0.05 vs. control, t-Test.

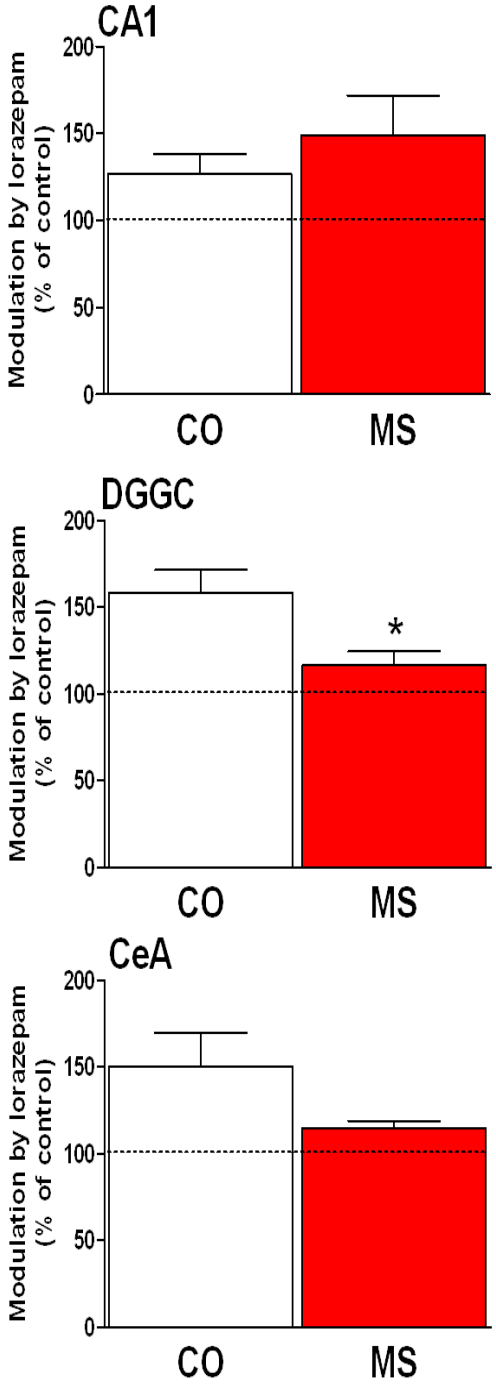

Fig.12: Effect of RMS on modulatory action of the benzodiazepine- lorazepam, on GABAergic currents at the level of CA1 area of hippocampus (CA1), dentate gyrus granule cells (DGGCs), and central nucleus of Amygdala (CeA). Values are expressed as % change vs. the respective control (response in the absence of drug). The numbers inside the bar indicated the value of "n". *$p<0.05$ vs. control, t-Test.

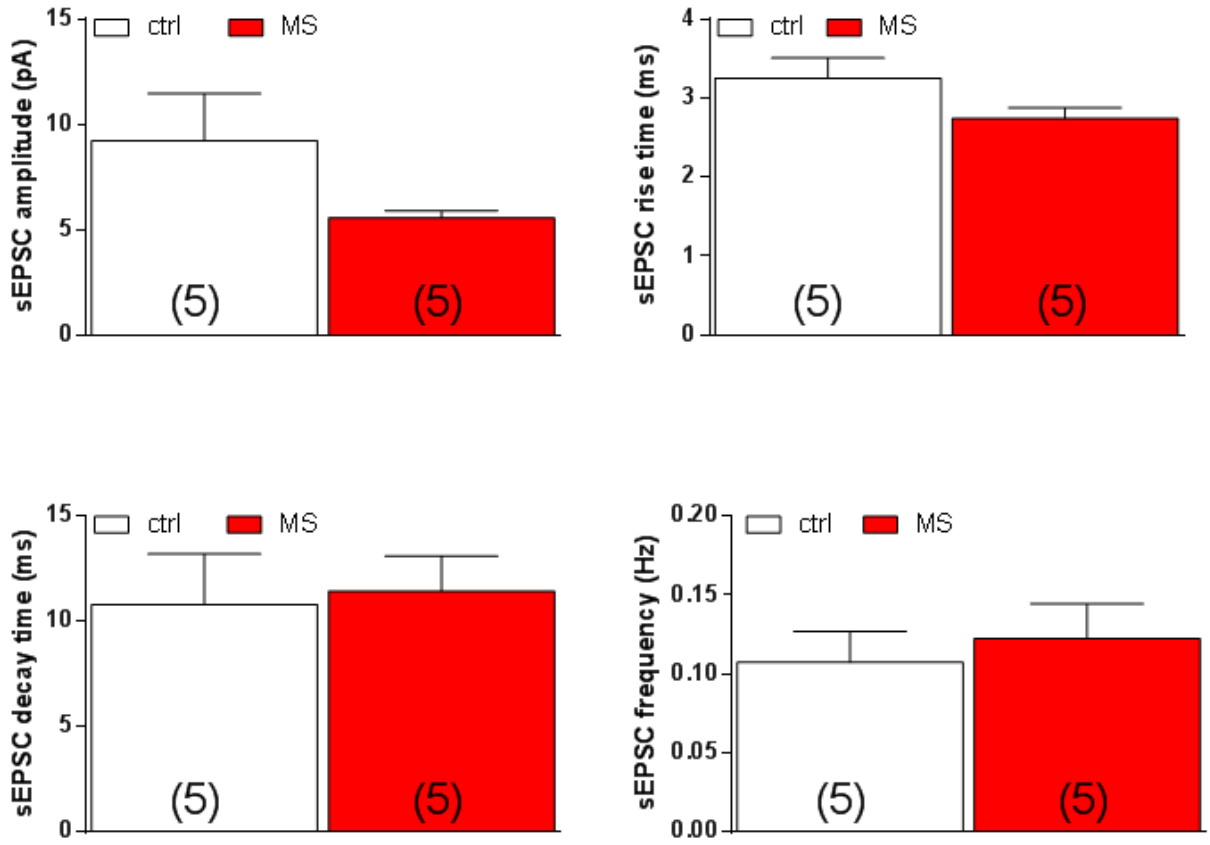

Fig.13: Effect of RMS on kinetic properties of basal sEPSC recorded pyramidal neurons of the CA1 area of the hippocampus, of C57BL/6J mice. Values are expressed in absolute values for the different parameters. The numbers inside the bar indicated the value of "n". *p<0.05 vs. control, t-Test.

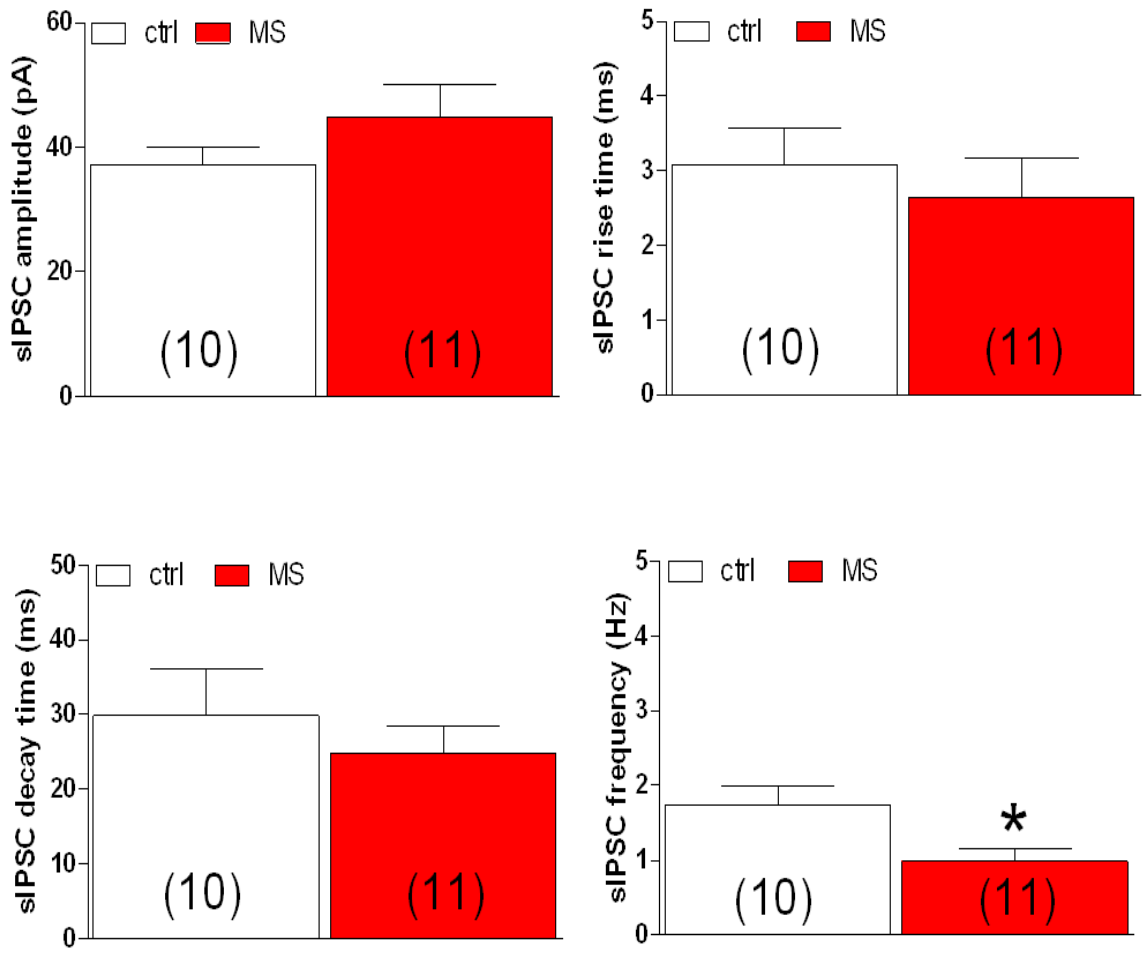

Fig.14: Effect of RMS on kinetic characteristics of basal GABAergic sIPSC recorded in the central nucleus of the amygdala. Values are expressed in absolute values relative to the different parameters. The numbers inside the bar indicated the value of "n". *p<0.05 vs. control, T-test.

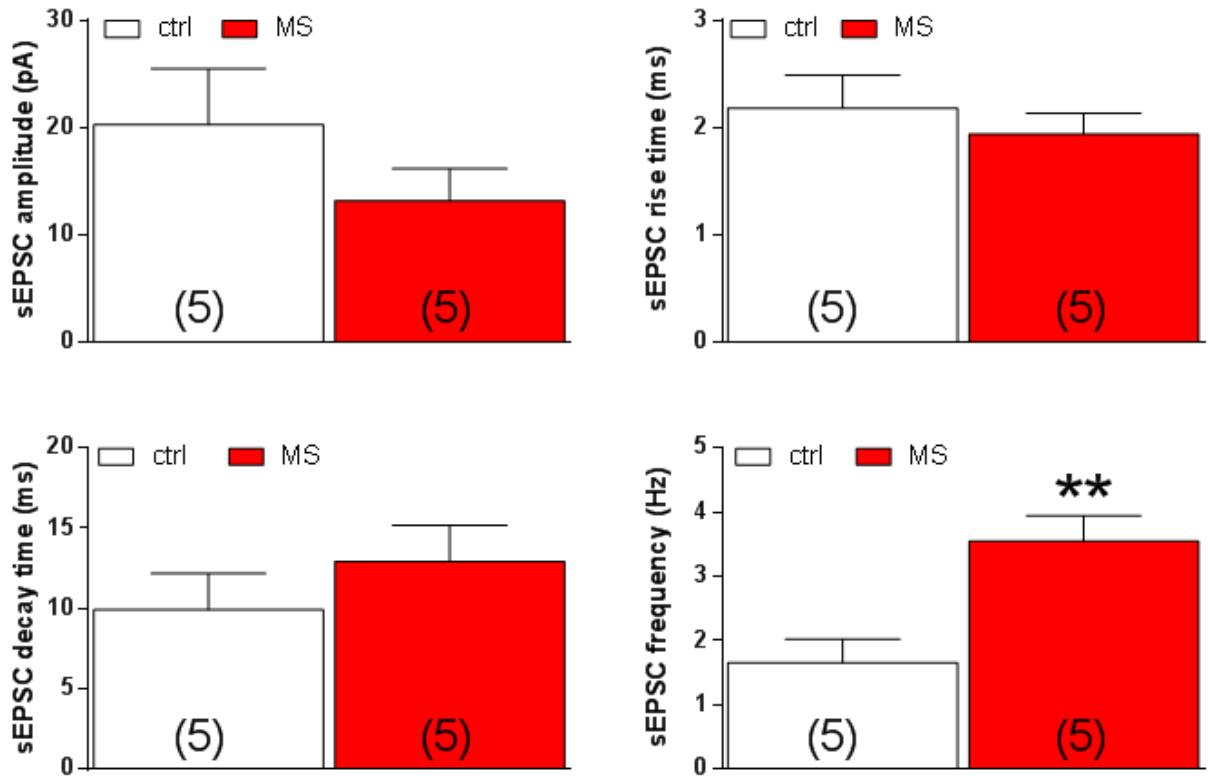

Fig.15: Effect of RMS on kinetic properties of basal sEPSC recorded pyramidal neurons of the area central nucleus amygdala. Values are expressed in absolute values for the different parameters. The numbers inside the bar indicated the value of "n". *p<0.05 vs. control, t-Test.

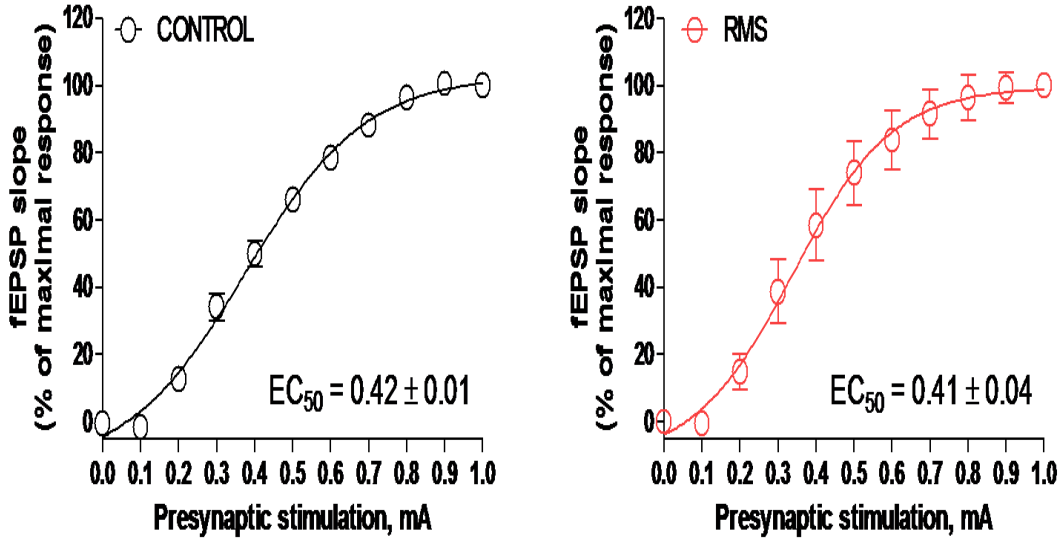

Fig.16: Effect of RMS on excitability of excitatory synapses CA3-CA1 in hippocampus. I-O curves were constructed considering the values of the fEPSP slope as a function of the intensity of the stimulation.

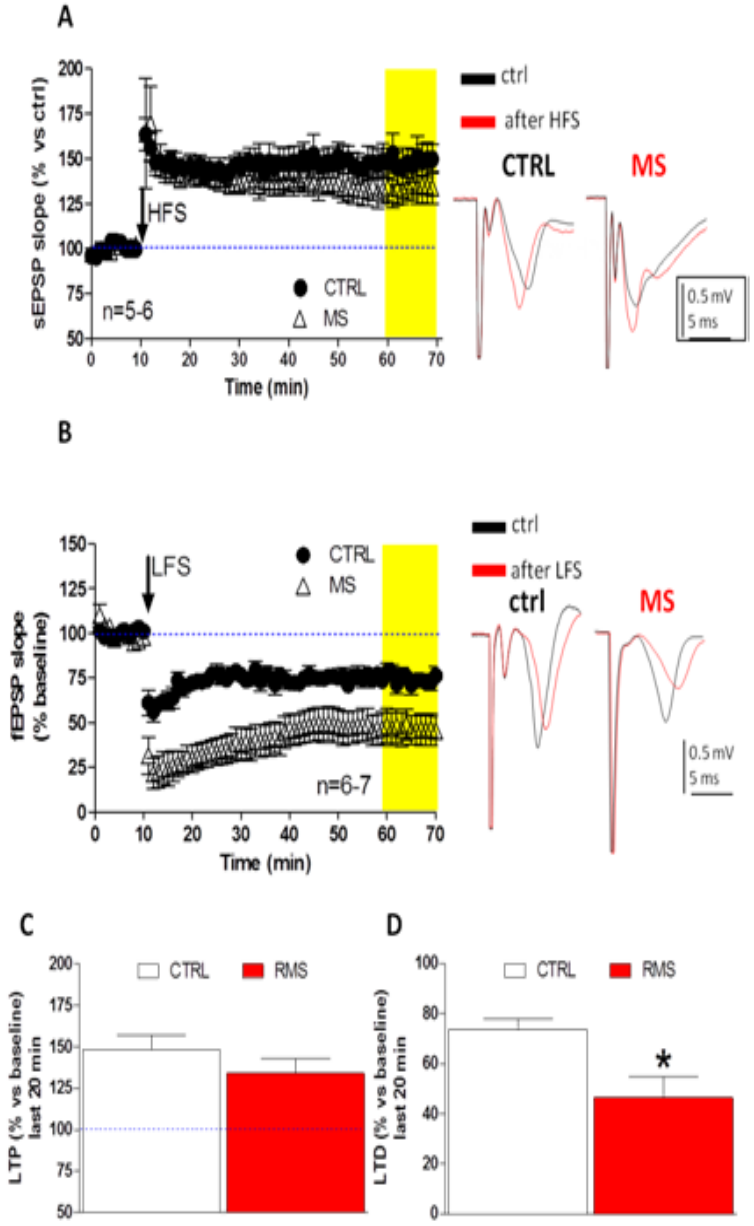

Fig.17: Effect of RMS on the different forms of synaptic plasticity (LTP, LTD) (A-D). **(A)** Long term potentiation **(B)** Long term depression. The data on the ordinate represents the percent change, from baseline, of fEPSP slope values, in the stratum radiatum (dendritic area) in the CA1 region of C57BL/6J mice. The number of recordings is equal to 10 for controls and 8 for the RSM. **(C)** The histogram of the averages of fEPSP (slope) for the last 10min recording (of about 1 h post-HFS). **(D)** The histogram of the averages of fEPSP (slope) for the last 10min of recording. (of about 1h post-HFS). t-Test, *p>0.05