TRACE ELEMENTS DISTRIBUTION IN THE BRAIN OF STRESSED RATS

**[1]Zerrouk Kamal, [2]Mohamed Najimi, [2]Mohammed Chigr,
[1]Mariama El Ouahli, [1]Ghizlane Er-Raoui and [1]Fatiha Chigr**

[1]Genie Biologique Laboratory, Faculty of Sciences and Techniques,
[2]Département of Chemistry and Environnement, Faculty of Sciences and Techniques,
Sultan Moulay Slimane University, Béni-Mellal, Morocco

ABSTRACT

Because of their ability to modulate the Gamma Aminobutyric Acid (GABA) a receptor complex, the principal aim of the current work is to assess two essential heavy metals: Iron (Fe) and zinc (Zn) by atomic absorption in different brain areas of stressed rats. To do so, we investigated the effect of acute immobilization stress (single 1-hour session) on the distribution and the densities of $GABA_A$ receptors as well as the concentrations of Zn and Fe in several rat brain regions of the stressed rats. Animals were randomly assigned to either control or stress conditions and changes in specific binding of the $GABA_A$ receptor as labelled with T-Butylbicyclophosphonothionate (TBPS) (ligand useful for $GABA_A$ receptor) were assessed by in vitro quantitative autoradiography with the aid of a computer-assisted image analysis system whereas the assessment of Fe and Zn concentrations was done by atomic absorption spectrophotometry. Exposure to 1h immobilization stress led to a significant increase in [^{35}S]-TBPS binding site density in stressed rats compared to controls (30-40% increase in cortex, hypothalamus, hippocampus and substatntia nigra). In the other analyzed brain structures, specific binding of [^{35}S]-TBPS remained unchanged in stressed rats. The spectrophotometer analysis showed significant decrease in Zn levels in the whole forebrain structures as well as the mesencephalon of stressed rats. The striking differences are noticed in hippocampus and mesencephalon. Furthermore, Fe endogenous concentrations display similar pattern following stress. The present study demonstrates that immobilization stress induces an increase in $GABA_A$ receptors concomitant to a reduction of Zn and Fe content in the stress sensitive rat brain structures. Besides supporting the alteration of the modulatory function occurring at the $GABA_A$ receptor level after stress, our data also reveal that the measured brain concentrations of the investigated heavy metals remain not sufficient to efficiently modulate the activity of with efficacy such complex receptor. This could explain the higher densities of $GABA_A$ receptors observed after acute stress.

Keywords: Zinc, Iron, $GABA_A$, Stress, Brain

1. INTRODUCTION

Gamma Aminobutyric Acid (GABA) A receptors are ligand-gated ion channels, mediating fast synaptic inhibition in the Central Nervous System (CNS) (Mody, 2012; Farrant and Nusser, 2005). These receptors are the targets of a variety of pharmacologically and clinically important drugs such as benzodiazepines, depressant barbiturates and neuroactive steroids (Smith et al., 2007). Beside these major modulators, some metal cations are able to inhibit the GABA response of neurons in a variety of organisms (Chen and Yung, 2006). Among them zinc and at a lesser extent iron, have been reported to be the most potent (Rahman, 2007). Zn^{2+} ions inhibit $GABA_A$ receptor function by an allosteric mechanism that is critically dependent on the receptor subunit

Corresponding Author: Fatiha Chigr, Laboratoire de Génie Biologique, Faculté des Sciences et Techniques, PO. Box: 523,
Sultan Moulay Slimane University, Béni-Mellal 23000, Morocco Tel: 212-523485112 Fax: 212-523485201

composition: αβ-subunit combinations show the highest sensitivity to Zn^{2+} (Krishek *et al.*, 1998; Saxena and Macdonald, 1996). Zn^{2+} antagonism is also affected by α- subunit (Draguhn *et al.*, 1990; Smart *et al.*, 1991; White and Gurley, 1995). The inhibitory effect of Zn^{2+} is reduced by exchanging α1-subunit for α2- or α3-subunits. Finally, Zn^{2+} as picrotoxin and pregnenolone sulfate, two endogenous modulators of $GABA_A$ receptor, exert their effect directly binding in the ion channel (Hosie *et al.*, 2003; Krishek *et al.*, 1996). The mechanism involving the Gamma-Aminobutyric Acid (GABA) in stress is well known (Foddi *et al.*, 1997). Indeed, many reports suggest that $GABA_A$ receptors may mediate responses to stressors and the physiological control of stress (Drugan *et al.*, 1993; Gunn *et al.*, 2011).Stress has also been shown to be associated with alterations in the capacity of ligand binding to the $GABA_A$ receptor complex (Concas *et al.*, 1993; Barbaccia *et al.*, 1996; Serra *et al.*, 2000). $GABA_A$ receptors density and modulation have been documented to significantly increase in density following the application of acute stress (Chigr *et al.*, 2001a; 2001b; 2002) probably as a consequence of an alteration of endogenous modulator levels. recently, several stress paradigms have been shown to induce significant reduction in serum zinc concentration (Cieslic *et al.*, 2011). Fe^{2+} was also reported to interact with Zn^{2+} especially in stressful situations. Psychological stress may cause decreased Fe^{2+} absorption and iron redistribution in body resulting in low iron concentrations in the bone marrow, an effect counterbalanced by zinc (Li *et al.*, 2012). Therefore, the aim of the present study was to evaluate the changes that may occur for the concentrations of the two heavy metals Zn^{2+} and Fe^{2+} in the brain of stressed rats and to correlate such alterations to the [35S]-TBPS binding on the benzodiazepine/GABA chloride ionophore receptor complex.

2. MATERIALS AND METHODS

2.1. *In vitro* Autoadiography Procedures

The experiments were performed on male Wistar rats (n = 16, weighting initially 180-200 g). The animals were housed (3-4 per standard cage) under controlled laboratory conditions (12-h light/dark cycle, constant temperature: 22±2°C and humidity: 60±2%) with food and water freely available. These conditions are rigorously respected to avoid any other stress situations. All experimental procedures were conducted between 8:00 a.m. and 10:00 p.m. Animals were handled for 5 days (20 min day^{-1}) to habituate them to the stress of handling and assigned to either a control (no stress) (n =

8) or immobilization stress (one 1-h stress session) (n = 8). At the time of experiments, the rats of the experimental group were picked up from their cages and were immediately subjected to stress immobilization. Immobilization stress conducted under this protocol was done as previously described by Kvetnansky and Mikulaj (1970) with slight modifications. Stress was applied by placing the rats on a piece of wood and the front and hind legs of the rats were immobilized with plastic collars and adhesive tape on the boards of the wood. This allowed normal breathing and only restricted movements were possible (free movement of the head and tail). The animals were kept immobilized for 1 h. These experimental measures minimized the pain of the rats. Ten minutes after the single immobilization stress session animals were killed by decapitation. Control rats were killed after removal from the home cages without prior manipulations. In all experiments, the immobilization procedure and sacrifice were performed between 10 and 12 h, to avoid possible circadian variations in the receptor function. After sacrifice, the brains were removed, snap-frozen in dry ice and kept at -80°C until sectioning. Serial coronal 20 um thick sections from each brain were cut at -20°C in a cryostat (Figocut 2880, Reichert Jung), collected onto 2% gelatin-coated slides and kept at-20°C until used. [35S] T-Butylbicyclophosphonothionate (TBPS) autoradiography was conducted as we previously described (Chigr *et al.*, 2002). Briefly, after a preincubation step of 30 min in 50 mM Tris-HCl, pH 7.4, brain slices were incubated with 3 nM [35S]TBPS (100-140 Ci/mmol; NEN, Boston, MA) in 50 mM Tris-HCl buffer, pH 7.4, containing 500 mM NaCl and 10^{-4} M ascorbic acid, for 3 h at room temperature, in the absence (total binding) or in the presence of the unlabelled compound (picrotoxin, Sigma Chemical Co., St Louis, MO). The slices were then rinsed, dried out in ambient air, transferred to cardboard film cassettes along with slides containing labelled plastic standard (Amersham, Les Ulis, France) and apposed onto [3H]Hyperilm (Amersham). After an exposure period of 36 h at 4°C, the films were developed and fixed. The obtained autoradiographic labelling was quantified in many brainstem structures by computerized densitometry (Biocom, Les Ulis, France). Data were measured in relative Optical Density (OD) units for displacement studies. Drug-inhibited [35S]-TBPS binding was then plotted as a percentage of the specific binding obtained in the absence of the drugs. For non-displacement studies, the optical densities of each structure, were measured from the exposed film and converted into femtomoles/mg (fmol/mg), based on calibration curves of standards with known radioactivity (Miller, 1991). Specific binding was defined as total binding minus non-specific binding (binding in the

presence of 10^{-5} M picrotoxin). For all experiments, each point was the mean of measurements in five to eight sections for each individual animal, repeated with at least ive animals for either control or stressed rats. The data were compared by Analysis Of Variance (ANOVA) followed by Scheffe's test. A P value of <0.05 was considered statistically significant.

2.2. Brain Zinc and Iron Determination

For zinc and iron determination, two other groups of rats (n = 6, for each group) assigned to stress or not, were euthanized by decapitation 24 h after the session of stress. Rat brains were then collected and dissected to obtain: frontal cortex, hippocampus, thalamus, hypothalamus and mesencephalon the brain regions dissected were frozen and stored at-20°C until analysis. Each sample was wet-digested with nitric acid and hydrogen peroxide (microwave digestion). The zinc and iron concentrations were determined using flame atomic absorption spectrometry. The equipment used was a Thermo scientific, ICE 3000Spectrophotometer with deuterium background correction (air flow-4.2 L min^{-1}, acetylene flow-1.2 L min^{-1}, analytical wavelength-213.9 nm). Relative Standard Deviation (RSD) of the method (the whole analytical procedure: digestion + metal determination) did not exceed 2.4%. Mean recovery of zinc and for iron was 99%. The aqueous standard solutions were obtained from Zn (Zinc Standard Solution, Merck, Germany) and FeNO3 (Sigma Aldrich). The data obtained were analysed by Analysis Of Variance (ANOVA) followed by Scheffe's test. A *P* value of <0.05 was considered statistically significant.

3. RESULTS

The distribution of GABA$_A$ receptors, using [^{35}S]-TBPS as a ligand, showed a heterogeneous distribution in both stressed and unstressed rat brain. However, the quantification of the radioautographic labeling revealed the presence of significant differences in the density of [^{35}S]-TBPS binding sites. As shown in **Fig. 1**, high significant densities were principally found in the forebrain of stressed rats (30-40% increase). This includes hypothalamus, hippocampus and cerebral cortex. Detailed analysis, demonstrates that significant differences were confirmed in areas known to be sensitive to stress, such as layer IV of the cerebral cortex, the dentate gyrus in hippocampus and the paraventricular nucleus in the hypothalamus. In all these structures, the [^{35}S]-TBPS binding (2 nM) was increased significantly (p<0.05, n = 8 for each group and 6-8 measurements for each rat) after 1 h session of immobilization stress. The other structures belonging to cerebral cortex (as layerVI), thalamus (for example paraventricular nucleus) and hypothaIamus did not display any significant differences in TBPS binding. In the brainstem, the only striking difference was observed in the substantia nigra at the mesencephalic level in accordance with our previous results (p<0.05, n = 8 for each group and 6-8 measurements for each rat) following 1 h of immobilization stress session) (Chigr *et al.*, 2001a; 2001b). The binding observed in other structures as collucili and substantia gelatinosa was also not affected after stress (**Fig. 1**).
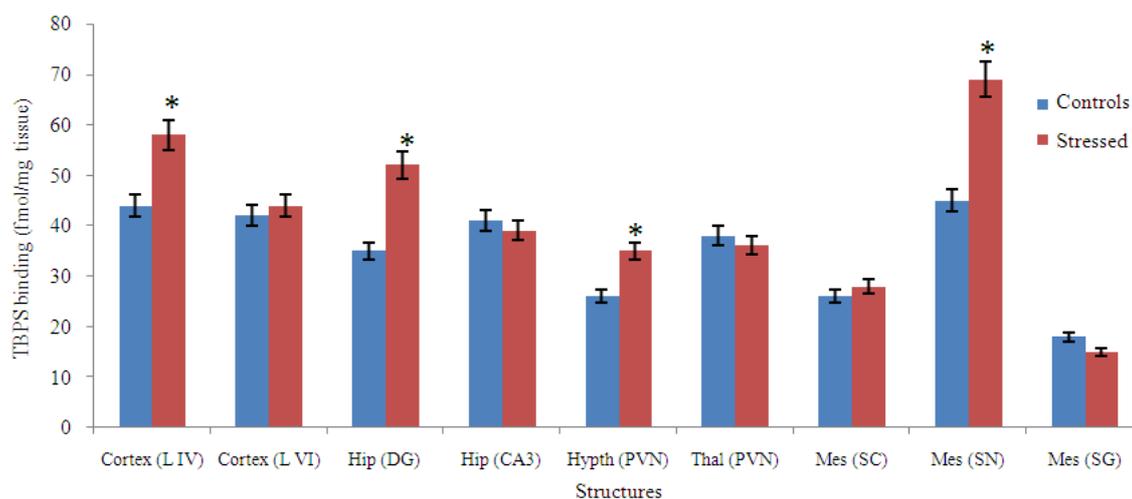

Fig. 1. Effect of 1h-immobilization on TBPS binding sites in different brain structures as determined by *in vitro* quantitative autoradiography
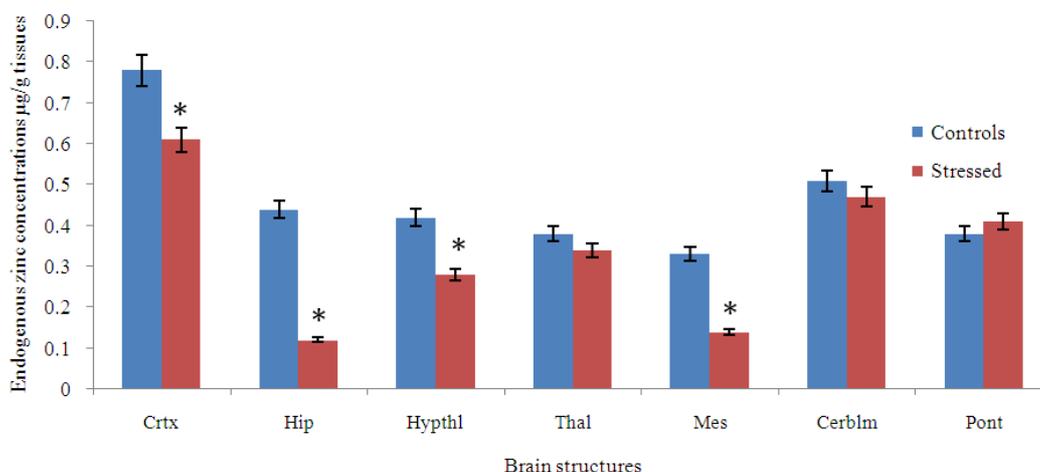
Fig. 2. Effect of 1h-immobilization on endogenous zinc concentrations in different brain structures as assessed by atomic absorption spectrophotometry
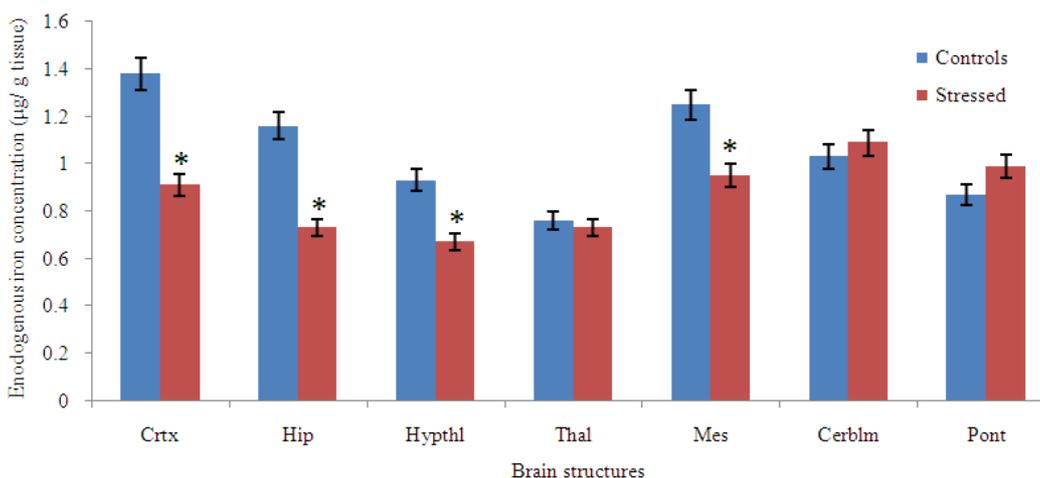

Fig. 3. Effect of 1h-immobilization on endogenous iron concentrations in different brain structures as assessed by atomic absorption spectrophotometry

In other stressed and unstressed rat groups (n = 6, for each group), we in parallel measured the endogenous brain levels of zinc and iron. The spectrophotometric analysis showed that the mean concentrations of Zn^{2+} are significantly decreased in all the sensitive structures reported to be sensitive to stress (displaying high TBPS binding). This effect on endogenous zinc concentrations is well displayed in forebrain structures, principally hippocampus; where the lowest concentrations were observed (25-65% decrease in stressed rats compared to controls, p<0.05, n = 6 for each experimental group; **Fig. 2**). A significant decrease in Zn concentration was also measured in the mesencephalon (around 55% of decrease in stressed rats compared to controls, p<0.05, **Fig. 2**). Thalamus, cerebellum and pontine which did not display increase in TBPS binding, also did not show any significant fluctuation in zinc concentration. Fe levels analysis showed significant decrease in the forebrain region and mesencephalon of the stressed rats (20-35% decrease in stressed rats compared to controls, compared to controls, p<0.05, **Fig. 3**). Similarly to endogenous zinc concentrations, stress does not affect significantly the endogenous iron concentrations in cerebellum and pontine (**Fig. 3**). Globally, the brain areas sensitive to stress, show both alterations in $GABA_A$ receptors and zinc and iron concentrations.

4. DISCUSSION

The present study examined the effects of immobilization stress on the brain concentrations of two essential heavy metals, i.e., zinc and iron, i.e., zinc and iron proposed as endogenous modulators of the GABA$_A$ receptor complex (for review Rahman, 2007). We firstly assessed the influence of the stress paradigm we used on the in vitro autoradiography [^{35}S]-TBPS binding, a ligand that binds at or near the chloride channel of the GABA$_A$ receptor complex (Atack et al., 2007). The experiments presented herein revealed that the GABA$_A$ receptor complex as labeled by [^{35}S]-TBPS was affected by a single exposure to stress (1-h immobilization) in the rat brain. These findings also show that stress effects were specifically noticed at specific regions of the rat brain. Indeed, some regions of the cerebral cortex, hippocampus, hypothalamus and substantia nigra were the structures sensitive to stress immobilization. No significant differences were observed in the other structures. Thereafter, we investigated the effect of acute immobilization stress on the endogenous metals concentration and demonstrated that a significant decrease in their brain levels. Unfortunately, while the autoradiography technique allows the fine anatomical determination of structures concerned by stress, this is not possible with spectrophotometry absorption technique that necessitates important brain punches. Thus, it was impossible to determine the Zn values for the layer IV of cerebral cortex or for the paraventricular nucleus of the hypothalamus. The values of metal concentrations concern the whole structure. This could not allow us to determine if other subdivisions in these structures account for the decrease observed or not.

The findings concerning stress effect on GABA$_A$ receptors density reinforce the idea that the GABAergic system seems to be differentially affected by stress according to the brain area studied (Losada, 1989). The enhancement of [^{35}S]-TBPS binding could be explained by the reduced concentrations of endogenous GABA, occurring specifically after stress. Such responses may, in turn enhanced the GABA$_A$ receptor sensitivity to other endogenous ligands. In addition, the present findings are different from previous data showing decreases in the binding of [^{35}S]-TBPS in many brain structures after stress (Drugan et al., 1993). The discrepancy may be due to several procedural factors or to the stress type applied. The mechanism whereby stress alters TBPS binding sites also allows other hypotheses. Thus, it is possible that the individual brain region differences observed could be due to the subunit heterogenous distribution in these brain structures (Fritschy and Mohler, 1995). As a consequence, the potency and magnitude of the stress effect could be influenced by the subunit composition of the GABA$_A$ receptor. In this sense, the selectivity of the stress effect for certain sub-unit assemblies could not be excluded. The observed alterations might also result from changes in brain neuroactive steroid levels known to exert anxiolytic (Bitran et al., 1991) and anti-stress (Barbaccia et al., 1996) effects in brain. It is likely that by reducing the synthesis and the release of neurosteroids, acute stress will enhance the [^{35}S]-TBPS binding. Finally, it is not excluded that the observed changes could be due to the fact that in the stress paradigm used, the concentration range over which several modulators were able to counteract the impairment in GABAergic transmission (Barbaccia et al., 1996; Concas et al., 1998) has not yet been reached in the brain structures sensitive to stress. This could explain why the low concentrations of zinc obtained after stress, are not able to efficiently inhibit GABA$_A$ receptor function. Indeed, the transition metal ion Zn^{2+} is concentrated into zinc-containing neurons in the CNS and is thought to be involved in modulating the sensitivity of GABA and glutamate receptors to their respective neurotransmitters (Frederickson, 1989; Frederickson and Bush, 2001). Zinc is known to inhibit and to reduce the spontaneous and the miniature inhibitory postsynaptic currents (Chen and Yung, 2006). The decrease in zinc concentrations leads probably to the alteration of the capacity of the metal to modulate of the tonic GABAergic inhibition (Smart et al., 2004). Among possible other explanations for this negative result is that synaptically released Zn^{2+} is confined to excitatory synapses and that Zn^{2+} spillover to GABAergic synapses is not sufficient to modulate the GABA$_A$ receptors present (Ruiz et al., 2003). Zinc as other active endogenous modulators of the GABA$_A$ receptor, is negatively affected by stress which reduces its endogenous concentrations and could results probably in uncoupling of zinc and TBPS binding sites in the GABA$_A$ receptor complex. As a consequence, the affinity of zinc towards its binding site in the GABA$_A$ receptor complex will decrease. Finally it is not excluded that some of these changes may be explained by altered expression of subunits that affect the sensitivity of receptors to Zn^{2+}. Zinc is known to exhibit anti-depressant like activity (Hosie et al., 2003) and any reduction in its endogenous levels leads to anxiety states (Huang et al., 2012). This is an agreement with the anxiety behavior observed in stressed rats (Charrier et al., 2006). Hence, supplementation of zinc was suggested to abolish or to reduce the anxiety-like state observed

following stressful conditions (Cope *et al*., 2011). The supplemented zinc will reach the containing zinc neurons to probably reinforce the role of the endogenous zinc. Zinc supplementation was also reported to prevent stress effects and stress-induced decrease in iron levels (Li *et al*., 2012). The data of the present investigations confirm low levels of endogenous cerebral iron after acute immobilization stress. This could be due to a changed systemic and brain iron homeostasis following the application of psychological stress (Ma *et al*., 2008; Wang *et al*., 2008) occurring via regulation of iron transport (Moss and Morgan, 2004). The role of transferrin seemed to be of high importance since stress induced reduction in ferritin and the subsequent reduction in iron storage and utilization (Huang *et al*., 2012).

Our findings and the mentioned reports support the fact that a reduction in endogenous level zinc disturbs iron homeostasis in brain, which seems to be highly sensitive to zinc levels. The reduction in serum zinc concentration has also been reported following stress which is concomitant with a decrease in BDNF expression in hippocampus (Cieslic *et al*., 2011). Indeed, zinc treatment has been shown to induce cortical BDNF gene expression (Nowak *et al*., 2004). The mechanism, by which the reduction of Zn influences that of BDNF expression, passes probably via the GABAergic system. GABA is reported to diminish the glutamate-stimulated expression of BDNF transcripts and inhibited the stimulatory effect of glutamate on BDNF peptide content (Marmigere *et al*., 2003). The alteration of both $GABA_A$ receptors and the two heavy metals in substantia nigra is of high interest, as it is a component of the basal ganglia, known to be involved principally in motor control. Furthermore, substantia nigra receives GABA projections from globus pallidus (Erlij *et al*., 2012). Any alteration in this circuit impaired locomotor behavior and locomotor activity. Interestingly, the impairment of these functions occurs also following stressful situations (Tamburella *et al*., 2012). Thus, the alterations observed in substantia nigra in this study, could explain these stress-induced motor dysfunctions and suggest that modulatory function of $GABA_A$ receptor by heavy metals could be important for basal ganglia functions. The effect of stress seemed to be specific, as the other parts of basal ganglia did not show any difference in TBPS binding and Zn and Fe concentrations (data not shown).

Taken together, these results support the alteration of the modulatory function occurring at the $GABA_A$ receptor level after a single session of immobilization stress. The concentration of the heavy metals investigated is too low to efficiently modulate the $GABA_A$ receptor function. This could explain the higher densities of $GABA_A$ receptors observed after acute stress. Finally, these investigations pointed the potential role played by essential heavy metals in plasticity processes related to the stress response and in the regulation of the homeostasis.

5. CONCLUSION

Immobilization stress induces up regulation of $GABA_A$ recptors which is concomitant to down regulation of endogeneous zinc, a potent modulator of this receptor as well as endogenous concentrations of iron in brain structures sensitive to stress. Wheteher the alteration of the two heavy metals concentration could be considered as a consequence or the cause of $GABA_A$ receptor dysfunction, other investigations are needed to elucidate these findings.

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