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Consequences of early adverse life events on GABAergic neurons produce long term changes in brain function

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ABSTRACT

Exposure to early stressful adverse life events may increase vulnerability to psychopathology in adult life. There are important memory disturbances in stress-related psychiatric disorders. Several investigations have demonstrated its impact both on the activity of the hypothalamic-pituitary-adrenal axis (HPA) and the development of psychoemotional disorders later in adult life. The lifespan of the predominant inhibitory neurotransmitter in the central nervous system (CNS): γ-aminobutyric acid (GABA), is determined by its uptake into neurons and glia, through high-affinity Na^+/Cl^- dependent transporters (GATs). The aim of this research was to evaluate the effects of acute and chronic maternal separation (AMS and CMS) plus cold stress from frontal cortex (FC) and hippocampus (Hic) on the expression levels of GAT-1 using immunoblotting whose appearance correlates with the plasmatic corticosterone levels at different postnatal days (PD) from birth to adulthood. To explain these phenomena, we used Western blotting to evaluate the alterations in the expression of Hic and FC GABA transporter proteins. In response to AMS + cold stress in FC we found that a decrease expression of GAT-1 at PD13. On the contrary, in CMS + cold stress we have demonstrated an increase in the levels of the expression of GAT-1 both at PD57 and PD63. In Hic, AMS + cold stress increased the levels of GAT-1 expression both at PD7 and PD13. When we study CMS + cold stress we showed a decrease at PD57 and PD63 meanwhile an important increased at PD71 of hippocampal levels expression of GAT-1. With respect to the levels of corticosterone we found an increase in all age groups studied in AMS. Conversely, we have seen a decrease in corticosterone concentrations except at PD71 in CMS. In summary, low responsiveness of the early postnatal period to stress are involvements of GABAergic system, suggesting that GATs may contribute to the deregulations of neuronal excitability that accompany neurobiological consequences of early stress: schizophrenia, epilepsy, ischemia, anxiety and depression. Our results serve as a starting point elucidating the molecular mechanism of transporter regulation in GABA pathways during postnatal development.

KEYWORDS: development plasticity, early maternal separation, acute and chronic stress, western blot, GAT-1, corticosterone levels

1. INTRODUCTION

The physiological response begins with activation of the hypothalamic-pituitary-adrenal axis (HPA), the autonomic nervous system and the immune system whose physiological mediators are the glucocorticoids (GC), catecholamines and cytokines respectively [1, 2, 3, 4]. All events begin promoting cellular adaptive changes in cells and tissues that protect the body and promote their survival.

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Different protocols of maternal separation (MS) in rats showed alterations in the functioning of the central nervous system (CNS) such as learning disabilities, voluntary alcohol intake and behavioral changes [5]. It is believed that the effects of these early life events are mediated by the high plasticity of the developing CNS [6, 7]. The neonatal period from days 3-14 has been described as a "stress hyporesponsive period" that is critical for the maturation of the HPA axis [8]. During the neonatal period, mothers provide tactile and chemical stimuli to their offspring that are necessary to proper pups´ HPA development and function. Consequently, acute or repeated long-term separation from the dam is one of the most potent naturally occurring stressors to which rat pups can be exposed during the "stress hyporesponsive period" [8].

While GABA is the main inhibitory neurotransmitter in the adult brain, GABAergic transmission is excitatory during early postnatal development. This different action of GABA results from a reversed chloride concentration gradient with higher intracellular chloride concentration in immature neurons [9, 10, 11, 12]. The GABA driving force is strongly depolarizing during the first postnatal week [13, 10, 11, 5, 14]. Changes in GAT activity are relevant both in pathology and therapy [15], an example being the GABA transporter inhibitor tiagabine, which is currently used as an antiepileptic drug [16]. The tight control of the extracellular levels of GABA is crucial for the right function and development of the central synapses and neural circuits. The extracellular concentrations of this inhibitory amino acid are largely regulated by transporter proteins expressed in the plasma membrane of both neurons and glial cells. GABA uptake is carried out in a highly selective Na^+-dependent manner [17, 18, 19, 20]. The most copiously expressed GABA transporter in the brain is GAT-1 [21].

The aim of the present work was to investigate the consequences of AMS or CMS plus cold stress on the pattern of expression of GAT-1 and its correlation with corticosterone levels during development and adulthood.

2. MATERIALS AND METHODS

Male Wistar rats were studied at different ages in AMS or CMS + cold stress. They were housed under constant temperature and a 12-hour light-dark cycle. They were kept in an acclimatized animal room (21-23ºC) with *ad libitum* access to dry food and tap water. All animal procedures were performed in accordance to our institutional guidelines after obtaining the permission of the Laboratory Animal Committee and with the U.S. National Institute of Health Guide for the Care and Use of Laboratory Animals (NHI publication N*8* 80-23/96). All efforts were made to minimize suffering of animals and to reduce the number of animals used.

Rat pups were separated from their mother from 5, 7, 13 or 21 PD for 1 h [22]. For this procedure, the pups were removed from their mother and then they were placed into fridge at 4ºC during 1 hour. Animal models are quite useful for elucidating the mechanisms underlying abnormalities toward possible treatment strategies for psychiatric diseases [23], emotional or neurological diseases [24]. Control pups were left undisturbed with their mothers. All pups were weaned on day 21. Maternal separations were carried out between 8:00 and 14:00 hs. These animals are the most desirable control group for investigations of the effects of MS. After weaning, rats were housed in same sex groups under standard conditions. **1-AMS+ stress:** a) Control group: neonates remained with their mother. b) Stressed group: neonates from 5, 7, 13 and 21 PD were removed from their mother and exposed to cold stress (4ºC) for 1 h. **2-CMS+ stress:** a) Control group: neonates remained with their mother until weaning at 21 PD. b) Stressed group: neonates from 5, 7, 13 and 21PD were removed without their mother and exposed to cold stress for 1 h/day for 20 days. After 20 days of stress, the animals remained for a period of 30 days in their cages (washout). Immediately after acute stress or 30 days or after chronic stress (55, 57, 63 and 71 PD), the animals were killed by decapitation.

The brains were removed from the cranial cavity: FC and Hic were dissected on a Petri dish at 0ºC, according to Glowinski and Iversen [25]. Protein content was estimated by the Lowry technique [26] using bovine serum albumin as the standard. Western blotting analysis was used to measure levels of GAT-1 expression in Hic and FC. Both areas were homogenized with a glass-PTFE homogenizer in lysis buffer (Tris Base 50 mM;

NaCl 150 mM; EDTA 2 mM; sodium dodecyl sulfate (SDS) 0.05%; Triton-X100 1%; Phenymethanesulfonyl fluoride (PMSF) 100g/ml; Leupeptin 1µg/ml). The protein concentration in the samples was analysed by the Lowry technique [26]. Samples of total proteins (20 µg) were separated on 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 130 V and transferred to nitrocellulose membrane using a blot system (Transblot, BioRad). The membranes were incubated in blocking buffer (1 X TBS and 5% non-fat dry milk) for 1 h at room temperature (RT) and thereafter incubated overnight at 4ºC with specific primary antibody: rabbit anti-GAT-1 (1:700), rabbit anti-actin (1:1000). Blots were then washed three times for 10 min in 1X TBS with 0.3% Tween 20 at RT and then incubated for 1 h at RT with goat anti rabbit IgG (1:2000) conjugated to horseradish peroxidase, then washed three times for 10 min in 1X TBS with 0.1% Tween 20 at RT. For quantification of band intensity, blots were scanned and analyzed using Image J PC software analysis. The expression level corresponds to the number of black pixels of each band. The results were expressed as optical density (OD) in arbitrary units. Actin was used as a loading control. The amount of protein was analysed as a ratio between the protein and actin, to ensure that an equal amount of protein was loaded onto the gel and transferred to the membrane.

Corticosterone plasma levels were determined in groups of animals (three to six animals per experimental group) at all ages studied in acute or chronic MS, by collecting trunk blood samples after decapitation. Plasma was separated by centrifugation and stored at -70ºC until high-perfomance liquid chromatography assay (HPLC) with electrochemical detection was performed according to Retana-Márquez *et al.* [27]. All blood samples were collected between 10:30 and 12:30 am.

All statistical analyses were performed with the GraphPadPrism 3.1, GraphPad Software Inc (San Diego, CA, USA). Differences were considered significant when P-value was lower than 0.05. Comparisons were carried out with one-way ANOVA (analysis of variance) followed by Tuckey post hoc test.

PMSF and Leupeptin were from Sigma Chemical Co., St. Louis, MO, USA. Kit ECL Western blotting substrate was from Pierce, Thermo Scientific IL-USA. Primary antibody: rabbit (AB1570) was from Millipore, Chemicon, USA; α-Actin, rabbit (A2066) was from Sigma-USA (G9269). Secondary antibody: α -rabbit-SIGMA was from Santa Cruz Biotechnology, INC. California, USA.

3. RESULTS

Western blotting was performed on homogenates prepared from either FC and Hic neonates or young adult rat brains. These blots reveal that the homogenates contain GAT-1 proteins (Figure 1 and 2). In frontal cortex AMS+ stressed rats' immunoblotting indicated a significant reduction in GAT-1 expression levels at PD13 (by 23%, $P<0.05$, Figure 1 A). On the contrary, in CMS+ cold stress increased the levels of GAT-1 at PD57 and decreased at PD63 (by 31% and 66%, $P<0.05$ respectively) (Figure 1B). When we studied the Hic, we observed that AMS+ cold stress produced a significant increment both at PD7 (by 290%, $P<0.01$) and PD13 (by 260%, $P<0.05$) (Figure 2A). Moreover in CMS plus cold stress we found a decrease at PD57 and PD63 (by 40%, $P<0.05$), and one important increase at PD71 (by 67%, $P<0.01$) in the levels of GLT-1 (Figure 2B).

Plasma corticosterone levels of both pups and young adults groups were measured immediately after AMS+ cold stress or 30 days after CMS plus cold stress. The main effects of corticosterone treatment we showed in AMS+ stress where corticosterone plasma increased in all ages examinated (Figure 3A) might be due to increase of maternal deprivation and cold stress. Furthermore, increased plasma levels for an hour AMS may be due to exposure to a novel environment. However, in rats stressed chronically without their mother we found a decrease at PD55 and PD57 ($p<0.05$), while we showed an increase at PD71 ($P<0.05$) (Figure 3B). This indicates that repeated early maternal separation can affect the regulatory mechanisms of adult HPA axis by acting in a period when the HPA axis itself is functioning poorly [28]. In addition, we think that although corticosterone has beneficial effect at short-term effects, at long-term corticosterone levels can result in damage to the physiological systems it protects acutely.

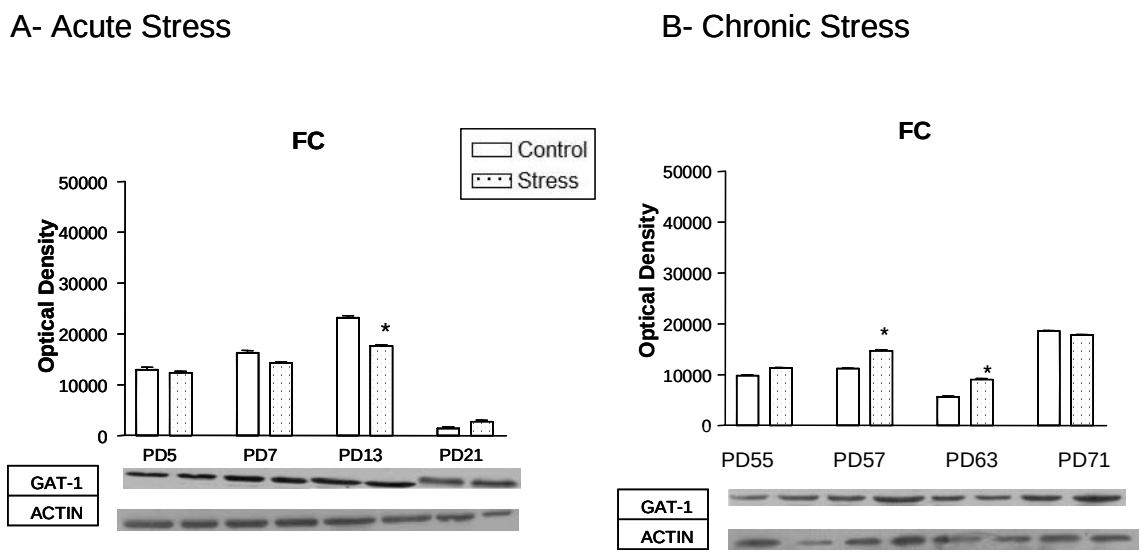

Figure 1. Western blot analysis of GAT (67 kDa) in FC homogenates from **A**: unstressed and acutely stressed rats. **B**: unstressed and cronically stressed rats. The expression level corresponds to the number of black pixels of each band counted using Image J. Actin (42 kDa) served as a loading control. Western blots are representative of 3 different experiments. OD: optical density, arbitrary units. *p<0.05, ** p<0.01 compared with the respectively control group.

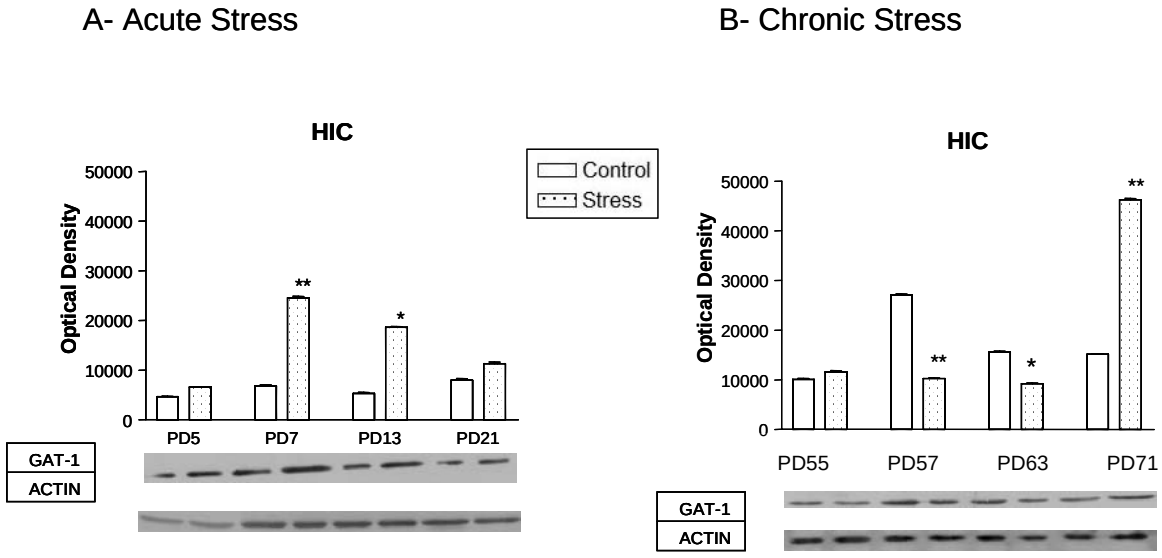

Figure 2. Western blot analysis of GAT (67 kDa) in Hic homogenates from **A**: unstressed and acutely stressed rats. **B**: unstressed and cronically stressed rats. The expression level corresponds to the number of black pixels of each band counted using Image J. Actin (42 kDa) served as a loading control. Western blots are representative of 3 different experiments. OD: optical density, arbitrary units. *p<0.05, ** p<0.01 compared with the respectively control group.

4. DISCUSSION

The present study shows that AMS and CMS plus cold stress affect the expression of GAT1 mediated GABA transport by nerve endings as well as corticosterone levels in limbic structures related to the regulation of HPA axis in a region-specific manner. Considering that the anterodorsal thalamic nuclei are a HPA regulatory region, functional studies

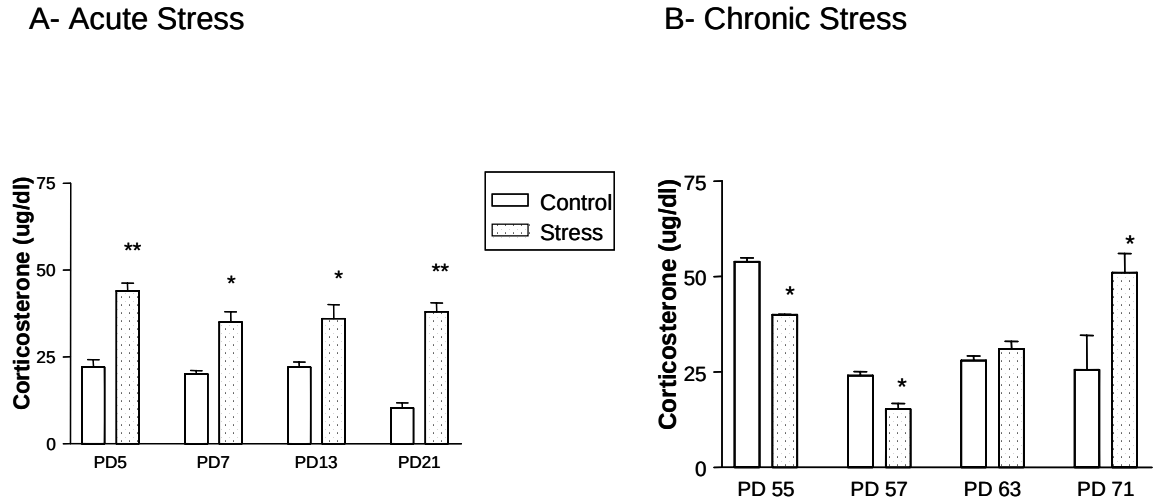

Figure 3. Plasmatic levels of corticosterone. The results are expressed in µg/dl. **A**: unstressed and acutely stressed rats. **B**: unstressed and cronically stressed rats. Values are the mean ± SEM (n=6-8). *p<0.05, ** p<0.01 compared with the respectively control group.

of this circuit would help us to better understand the role of these structures in stress integration [29]. In homogenates of FC and Hic acquired from either AMS or CMS + cold stress we found variations on the expression of GAT-1. GABA system plays a pivotal role in the pathophysiology of anxiety and mood disorders. The extracellular levels of GABA are regulated by specific high-affinity transporters, one of which, the plasma membrane GABA transporter-1 (GAT1), is considered the predominant neuronal transporter in the rodent brain [30, 31].

Our present data suggest that the Hic and FC also plays a role in terminating the HPA stress response. It appears legitimate to interpret this hypersecretion at the end of acute stress as evidence of an inability to terminate the stress response, rather than a prolonged perception of a stressor. Nevertheless, there was a decline in plasma corticosterone levels interpreted as evidence for the effects of control and predictability on the response of the HPA axis. We used 30-days washout in the chronic stress condition to check out if the effect of CMS plus cold stress persists as do the effects of early traumatic experiences in young adult.

Although AMS might mimic a ‘dramatic’ experience occurring at a precise developmental stage, the less dramatic repeated maternal separation can reproduce a more physiological situation [32].

CNS maintains a degree of adaptive plasticity, which allows it to adjust to certain conditions and modify the innate patterns of neuronal connections [33, 2]. These mediators exert a paradoxical damage-protection action. These variations can alter the functioning of the CNS and, consequently, the body's response to stress throughout life, as this treatment is done during the postnatal period, with the CNS in full development. A further support to this possibility came from the demonstration that prepulse inhibition disruption in maternally-deprived rats occurs only after puberty [34], with a temporal profile similar to the onset of schizophrenic symptomatology in patients, and was reversed by treatment with typical and atypical antipsychotic drugs [34], suggesting that the defects resulting from MS might be the consequence of an hyperactivity of the dopaminergic system.

The identification of neurobiological substrates that are affected by early life adverse experience may have important diagnostic implications and could contribute to identify novel molecular targets for the development of more effective treatments of psychiatric disorders. Further studies are now warranted to elucidate the type or the timing of early life events that are associated with enhanced risk for depression or anxiety may be different from those relevant to schizophrenia. The identification of the neurobiological substrates of

early adverse experience is of paramount importance for the development of novel treatments for children, adolescents and adults.

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