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IMPLICATION OF THE GABAERGIC SYSTEM IN ALTERED RESPONSES TO STRESS ASSOCIATED TO MATERNAL SEPARATION

Diploma thesis

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MOST USED ABBREVIATIONS

ACTH: adrenocorticotropin
BNDF: brain derived neurotrophic factor
BZDs: benzodiazepines
CNS: central nervous system
CREB: cAMP response element binding protein
CRF: corticotropin-releasing factor
CRH: corticotropin-releasing hormone
CSF: cerbrospinal fluid
DMS IV: Diagnostic and Statistical Manual
DNA: deoxyribonucleic acid
GABA: γ-aminobutyric acid
GABA-T: GABA-α-oxoglutarate transaminase
GAD: glutamic acid decarboxylase
GR: glucocorticoid receptor
HPA: hypothalamic-pituitary-adrenal axis
PND: postnatal day
MS: Maternal separation
NA: noradrenaline
PVN: paraventricular nucleus
5-HT: serotonin
WHO: World Health Organization

I. INTRODUCTION

1. DEPRESSION

1.1. Clinical epidemiology

Clinical depression, the phenotypic hallmark of the two leading mood disorders (major depressive disorder and bipolar affective disorder), is the most common psychiatric illness. According to the World Health Organisation (WHO) it yearly affects 121 million people worldwide, with 10–20% of women and 5 – 12% of men estimated to experience a depressive episode in any 1-year period, and with evidence of suicide rate of 15% of those affected (Choudary et al., 2005). The WHO/World Bank´s estimates of the global burden of disease, calculated in terms of disability-adjusted life years, found that unipolar depression was the leading cause of disability worldwide in the 15- 44-year age group, whereas bipolar disorder was the sixth most disabling disorder (Costello et al., 2002). Mood disorders are recurrent, life treatening (due to the risk of suicide), and a major cause of morbidity worldwide. At European level, the total (women and men) incidence of severe forms of depression is approximately 5%, while the incidence of milder forms is about 10% (European Study of Epidemiology of Mental Disorders/Mental Health Disability, 2000). According to the above mentioned, depression is almost twice as common in women than men (Nestler et al., 2002), and women have a two-fold-higher lifetime prevalence of major depression than men (Swaab et al., 2005). However, bipolar depressive disorder (also known as manic-depressive illness), afflicts both sexes equally and the incidence is roughly 1%. Prevalence estimates for mood disorders vary with the time period of reference (age) and method assessment (Costello et al., 2002) but actually it seems to be increased in adolescence (age 15-24) being reflected especially in anxiety disorders, hyperactivity or conversely in inattentiveness (Costello et al., 2002).

Depression should not be considered as a single disease, but a heterogenous syndrome consisting of numerous diseases of distinct etiology and pathophysiology. Epidemiologic studies show that roughly 40-50% of the risk to suffer from depression is genetic (Fava and Kendler, 2000; Sanders et al., 1999). This makes depression a highly hereditary disorder. In addition, nongenetic factors as diverse as stress and emotional trauma, viral infections (e.g. Borna virus), and even stochastic (or random) processes during brain development have been implicated in the etiology of depression (Nestler et al., 2002). All these factors contribute significantly to the inter-individual variations in the prevalence of this mood disorder. The

individual-specific environment, including family environment where the individual grew up, is considered to be one of the main factors conceivably involved in the development of depression.

1.2. Definition

Diagnostic criteria for major depression are depressed mood, irritability, low self esteem, feelings of hopelessness, worthlessness, and guilt, agresivity, decreased ability to concentrate and think, decreased or increased appetite, weight loss or weight gain, psychomotor retardation, insomnia or hypersomnia, low energy, fatigue, or increased agitation, anhedonia (decreased interest in pleasurable stimuli such as sex, food and social interactions), recurrent thoughts of death and suicide (Diagnostic and Statistical Manual, DSM IV, 2000), although all these symptoms may vary in intensity and duration.

A diagnosis of depression is made when certain number of the above cited symptoms are reported for longer than a 2 week period of time and the symptoms disrupt normal life functioning (Nestler et al., 2002). When depression is diagnosed, then an adequate treatment is necessary.

1.3. Classification of the main depressive syndromes

There are no definitive consenses on the classification of depressive syndromes due to heterogeneity of symptoms themselves. The following depression subtypes classification is based solely on symptomatic differences and there is as yet no evidence that it reflects different underlying disease states. In general depressive syndrome may be classified in accordance with the etiology, symptoms and evolution of the illness.

- According to the criterion of etiology:

Endogenous. Severe symptoms and prominent neurodegenerative abnormalities are observed. It is not possible to determinate the feature factor inducing the illness.

Reactive. Moderate symptoms are apparent in response to external factors. It is aroused by a clearly identified factor. However, the sadness, logic at the beginning, becomes pathological later because of its inadequacy in intensity and duration.

Organic. Accompanying other somatic illnesses.

- According to the description of symptoms:

Minor. Exhibits psychopatologic manifestations of milder intensity.

Major. Severe symptoms are present.

- According to the evolution of the illness

Bipolar. A complex genetic disorder in which the core feature is pathological disturbance in mood (affect) ranging from extreme elation, or mania (an enthusiasm inadequate to the circumstances), to severe depression usually accompanied by disturbances in thinking and behaviour (Craddock et al., 1999).

Unipolar. Only depressive states are present.

However, the classification proposed by the American Psychiatric Association, the Diagnostic and Statistic Manual (DMS IV) is currently one of the mostly accepted worldwide. Depressive disorders are classified into:

Major depressive disorder. Unipolar
Bipolar

Cyclothymic/dysthymic disorders.

Atypical disorders. Unipolar
Bipolar

1.4. Etiology of depression

1.4.1. Genetic factors

Within the past decade intensive molecular genetic research has been carried out attempting to determine chromosomal loci that may be linked to neuropsychiatric disorders (Martin, 1989). The search for specific genes that confer the risk to suffer from depression has been frustrating, with no genetic abnormality being identified yet to date with certainty. The difficulty in finding depression vulnerability genes parallels the difficulty in finding genes for other psychiatric disorders and, in fact, for most common complex diseases. There are many reasons for this difficulty including the fact that depression is a complex phenomenon with many genes possibly involved. Thus, any single gene might produce a relatively small effect and would therefore be difficult to detect experimentally. It is also possible that variants in different genes may contribute to depression in each family, which further complicates the search for depression genes (Nestler et al., 2002). Family, twin, and adoption studies provide an impressive and consistent body of evidence supporting the existence of genes determining predisposition to affective disorders, e.g. in bipolar disorder in which the approximate lifetime to suffer from this illness in relatives of a bipolar proband are: identical twin 40-70%; first degree relative 5-10%; unrelated person 0.5-1.5% (Craddock et al., 1999).

A "Mendelian disease" runs in families in a strict dominant, recessive, or X-linked fashion. In the late 1980s there were two high profile claims of X-linked fashion in the journal *Nature*: Baron et al reported linkage to X chromosome markers in several Israeli pedigrees and Egeland et al reported linkage to markers on chromosome 11p in a large pedigree of the Old Order Amish community in Pennsylvania. But in updated and extended analyses of their own data the significant evidence of linkage all but vanished (reviewed by Craddock). Hence, so far there are only very few examples of psychiatric illnesses (nor depression) inherited in a strictly Mendelian fashion (Burmeister, 1998). It is necessary to consider the following basic problems: a) incomplete penetrance, i.e., someone who carries the disease allele may not become ill, or the onset may be extremely late (possibly because of polygenic or environmental protective effects or variable age of onset); b) phenocopy, i.e., someone, even with relatives suffering from depression linked to a genetic background, may become ill for a nongenetic reason; c) heterogeneity, i.e., mutations in many different genes can have the same clinical outcome; d) polygenic inheritance, for example the additive effects of several

different alleles on quantitative traits such as blood pressure, or epistatic interaction, as suspected in schizophrenia and autism, where several predisposing alleles have to come together; e) high frequency of either the predisposing alleles or the disorder; and f) other genetic mechanism of inheritance, such as mitochondrial inheritance, or a genome that is actively changing, as in disorders with trinucleotide expansions (Craddock et al, 1999; Burmeister, 1998). Clinicians have always known that bipolar disorder tends to run in families but recent advances in molecular genetics nowadays provide the tools needed to identify genes influencing susceptibility. Four regions are attracting a great deal of interest in linkage studies of bipolar disorder, of these 12q23-q24 and 21q22 are, perhaps, the most promising.

DNA variation includes insertions and duplications, deletions and single-nucleotide polymorphisms (SNPs). SNPs are the driving force in psychiatric genetics (Prathikanti et al., 2005). Focusing on illnesses including mayor depressive disorder, Caspi et al demonstrated that a functional polymorphism in the promoter region of the serotonin transporter (5-HTT) gene, which effects transcriptional activity and expression of the transporter protein, modulates the influence of stressful life events on predisposition to depression. These authors came to three fundamental aspects of genetic risk for depression: 1) the gene itself does not cause depression, but biases the effect of negative environmental experience, a so-called gene–environment interaction; 2) effects of stress during early development may be especially effected by this genotype and finally 3) the nonrisk allele appears to confer some resilience to negative environmental experience (Prathikanti et al., 2005; Caspi et al., 2003).

In a further step, researchers have turned to the specificity offered by molecular biology techniques to study possible anomalies of chromosomes accountable for neurological and psychiatric disorders. Studies based on polymorphism of restriction fragments of DNA chain were performed. Its known that forehead fragments do not occur by chance but are hereditary. All this provide an useful marker to asociate a certain locus of DNA chain with a determinated disorder. It is also clear that genetically modified, knockout mice, can be used as pharmacological tools. In particular, knockouts can be used to assess whether selective or non-selective agonists require a specific receptor to be effective (Gingrich et al., 2001; Mayorga et al., 2001).

1.4.2. Monoaminergic hypothesis of depression

Tradicionally, affective disorders have been considered to be derived from impaired function of single or more neuronal pathways in the limbic cerebral region, especially in monoaminergic (noradrenergic and serotonergic) system. In its original form, the hypothesis proposed that depression was caused by a functional deficit of monoamines (noradrenaline, NA, and serotonin, 5-HT) at key sites of the brain, and that antidepressant agents exerted their effect by facilitating monoaminergic neorotransmission by increasing monoamine levels at neuronal synapses, as while mania was caused by a functional excess (Blier, 2002). The idea that biogenic monoamines are involved in the aetiology of depression came initially from three main lines of evidence. Firstly, drugs such as reserpine that cause depletion of brain monoamines can induce symptoms of depression; secondly, some depressed patients have reduced levels of monoaminergic metabolites in some body fluids, particularly in cerebrospinal fluid; and finally, drugs that relieve depression seem to atenuate the mechanisms by which 5-HT and NA are inactivated (Blier, 2002). However, the hypothesis could not explain the need of up to 2-3 weeks of continued drug treatment to alleviate depressive symptoms, even though monoamine levels increased within 1-2 days (Hindmarch, 2001). Moreover, conventional antidepressants are not effective approximately in 40% of pacients suffering from mayor depressive disorder or dysthimia (Shatzberg, 2000).

Therefore, it has been suggested that deficit of biogenic monoamine levels does not seem to be sufficient for the development of depression (Delgado et al, 1999). The fact that some drugs (e.g. cocaine and amphetamine) that enhance serotonergic or noradrenergic transmission are not effective in treatment of depression (Hindmarch, 2001) cannot be explained either by the monoaminergic hypothesis.

Researchers have kept searching for new hypothesis on etiology of depression, and biochemical changes implicating sensibilization and modulation of certain receptores have been suggested. It has been proposed that the temporal delay in clinical effects of antidepressants may be due to the time required for desensitization of the presynaptic 5-HT_{1A} and α_2-adrenergic autoreceptors which modulate 5-HT and NA release respectively. It has been also described that following the desensitization of α_{2C}-autoreceptors, α_{2A}-heteroreceptors and 5-HT_{1A}-autoreceptors after chronic treatment with antidepressant drugs, the negative feedback mechanisms operating on monoamine synthesis (rate-limiting enzymes)

might be less functional, which could allow nerve terminals to synthetize more NA and 5-HT. Furthermore, activation and desensitization of postsynaptic receptors after chronic antidepressant treatment could also play a role in explaining the delay in the therapeutic effect (Esteban, 1999).

Other investigators have emphasized on the long-term adaptive changes in brain areas modulated by 5-HT, and have suggested that increased 5-HT levels at the synaptic cleft are only the first step in a cascade of events leading to adaptive changes in neurons modulated by 5-HT, and these adaptive changes, not simply the restoration of 5-HT levels, would lead to the clinical improvement (Delgado, 1999).

Other possible examples of evolved changes are:

- reduced sensibility of adenyl cyclase accompanied by reduction of cortical β-adrenoreceptor density

- reduced density of serotonergic 5-HT_{2A} and α_2-adrenergic presynaptic receptors

- changes in density of GABA B receptors

1.4.3. Involvement of the hypothalamic-pituitary-adrenal axis

Chronic stress and stressful life events have been linked to the onset of depression and its severity (Hindmarch, 2001). A prominent mechanism by which the brain reacts to acute and chronic stress is activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1). Neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing factor (CRF), which stimulates the synthesis and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex. Glucocorticoids exert profound effects on general metabolism and also dramatically affect behaviour via direct actions on numerous brain regions.

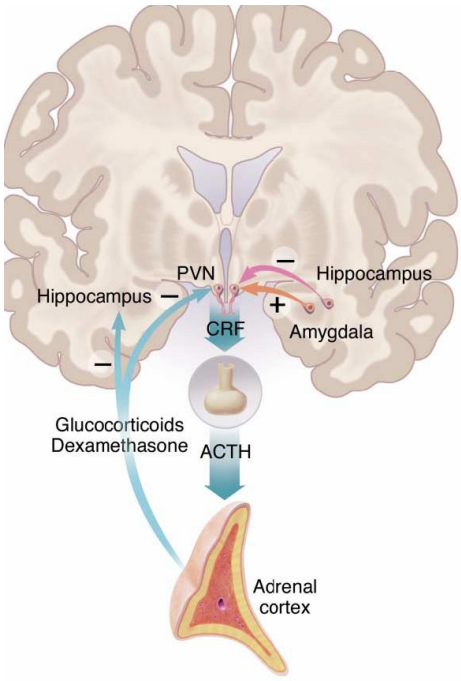

Figure 1. Regulation of the Hypothalamic-Pituitary-Adrenal Axis. CRF-containing parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) integrate information relevant to stress. Prominent neural inputs include excitatory afferents from the amygdala and inhibitory (polysynaptic) afferents from the hippocampus, as shown in the figure. CRF is released by these neurons into the hypophyseal portal system and acts on the anterior pituitary to release ACTH. ACTH reaches the adrenal cortex via the bloodstream, where it stimulates the release of glucocorticoids.

The activity of the HPA axis is controlled by several brain pathways, including the hippocampus and the amygdala (Figure 1). Glucorticoids, by regulating hippocampal and PVN neurons, exert powerful feedback effects on the HPA axis. Levels of glucocorticoids measured under normal physiological circumstances seem to be able to enhance hippocampal inhibition of HPA activity (Nestler et al., 2002). In addition to its many functions, glucocorticoids (including synthetic forms such as dexamethasone) repress CRF and ACTH synthesis and release. In this manner, glucocorticoids inhibit their own synthesis. In addition, reductions in expression of postsynaptic CRF receptors in frontal cortex in depressive patients were observed (Nemeroff, 1988). Under pathophysiological circumstances such as stress, glucocorticoids may even have deleterious effects on the hippocampus, which could initiate and maintain a hypercortisolemic state related to some cases of depression. Glucocorticoid receptors (GR, type II) expressed in the anterior pituitary gland and widely distributed throughout the CNS are involved in regulating the HPA axis via negative feedback mechanism, which appears to be critical in dampening down stress-induced activation of the

HPA axis and shutting off further glucocorticoid secretion. Therefore, it has been suggested that a primary alteration in GR number and function may contribute to the patophysiology of depression (Hindmarch, 2001). According to studies performed on humans, certain tricyclic antidepressants, electroconvulsive therapy and chronic treatment with desipramine modulate activity of GR type II (Holsboer, 2000; Rossby et al., 1995). However, sustained elevations of glucocorticoids, observed under conditions of prolonged and severe stress, may result in alterations of GR that become less functional or resistent to the action of glucocorticoids (Hansen-Grant et al., 1998). Stress may also damage hippocampal neurons, particularly CA3 pyramidal neurons. Impaired hippocampal function might then be expected to both contribute to some of the cognitive abnormalities of depression and to reduce the inhibitory control that the hippocampus exerts on the HPA axis, which would further increase circulating glucocorticoid levels and subsequent hippocampal damage. The precise nature of this damage remains incompletely understood, but may involve a reduction in dendritic branching and a loss of the highly specialized dendritic spines where the neurons receive their glutamatergic synaptic inputs (Figure 2). Stress and the resulting hypercortisolemia also reduce the birth of new granule cell neurons in the adult hippocampal dentate gyrus. Such hippocampal neurogenesis is proposed to contribute to memory formation, but this point remains controversial. In addition to immediate induction of morphological and structural changes in hippocampus and potencially in others cerebral regions, e.g. frontal cortex, stress and glucocorticoid excess reduce the cellular resistence by increasing of the vulnerabillity of neurons to cell death, such as ischaemia, hypoglycaemia or exposure to certain toxins (Gould et al., 1998).

Abnormal, excessive activation of the HPA axis is observed in approximately half of individuals with depression, and these abnormalities are corrected by antidepressant treatment. Antidepressants may reverse and prevent the actions of stress on the hippocampus, and ameliorate certain symptoms of depression by increasing the dendritic arborizations and BDNF expression of these hippocampal neurons (Figure 2).

Some patients exhibit increased cortisol production, as measured by increases in urinary free cortisol levels (Nestler, 2002). Hypercortisolemia is believed to be associated with resistance to glucocorticoid-mediated feedback, as indicated by the *dexamethasone supression test*, in which a single dose of dexamethasone (see Figure 1) suppress plasma concentrations

of cortisol to a lesser extent and/or for a shorter time in depressed patients compared with normal subject (Hindmarch, 2001).

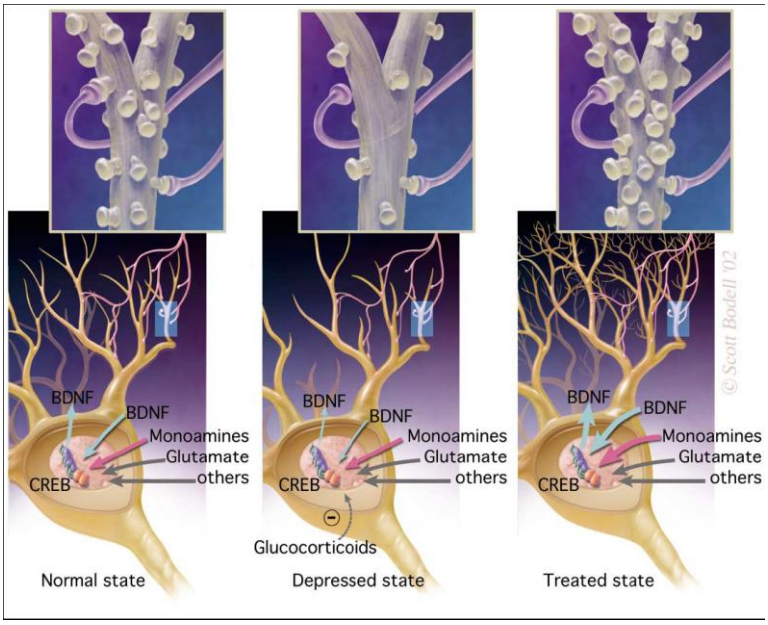

Figure 2. Neurotrophic Mechanisms in Depression**.** The panel above shows a normal hippocampal pyramidal neuron and its innervation by glutamatergic, monoaminergic, and other neurons. Its regulation by BDNF (*brain derived neutotrophic factor*, derived from hippocampus or other brain areas, likewise implicated in neuroprotection, cell survival and synaptic plasticity) is also shown. Severe stress causes several changes in these neurons, including a reduction in their dendritic arborizations, and a reduction in BDNF expression. The reduction in BDNF is partly mediated by excessive levels of glucocorticoids, which could interfere with the normal transcriptional mechanisms (e.g., CREB, *cAMP response element binding protein*) that control BDNF expression.

There also is direct and indirect evidence for hypersecretion of CRF in some depressed patients (Nestler, 2002). Other test of dynamic HPA function include the *CRF test*, in which ACTH and cortisol levels are monitored in response to a chalenge with intravenous CRF (Hindmarch, 2001). ACTH responses to intravenously administered CRF are blunted, and increased concentrations of CRF have been found in the cerebrospinal fluid.

There also are striking parallels between some aspects of the stress response, severe depression, and the effects of centrally administered CRF. These include increased arousal and vigilance, decreased appetite, decreased sexual behaviour, and increased heart rate and blood pressure. Moreover, increased concentrations of CRF were observed in cerebrospinal

fluid in certain depressive patients (Kling et al., 1991; Nemeroff et al., 1984). This has led to the proposal that a hyperactive HPA axis may contribute to depression not only via hypercortisolemism, but also via enhanced CRF transmission in the hypothalamus and other (extrahypothalamic) brain regions that are innervated by these neurons. Consistent with these human data are the experimental observations in animals, in which pups of rodents separated from their mothers early in life show abnormalities in HPA axis function, which resemble those seen in some depressed humans These abnormalities can persist into adulthood and be corrected by antidepressant treatments.

Despite the compelling model outlined above, it is still unknown whether HPA axis abnormalities are a primary cause of depression or, instead, secondary to some other initiating cause. Such observations have provided a clear rationale for the use of glucocorticoid or CRF receptor antagonists as novel antidepressant treatments. Intense attention is being given to the use of antagonists of the CRF_1 receptor, mostly expressed CRF receptor in brain, although agents directed against CRF_2 receptors are also of interest. CRF_1 receptor antagonists exert clear antidepressant-like effects in several stress-based rodent models of depression. These drugs may treat depression by limiting hypercortisolism through actions on the HPA axis (see Figure 1). Moreover, an action with potentially greater impact on depression, assuming the drugs prove clinically effective, may be inhibition of the CRF system in many other brain regions, independent of the PVN and the HPA axis. For example, in the amygdala and several related brain areas, CRF is a critical mediator of fear conditioning and other forms of emotional memory to both aversive and rewarding stimuli. Likewise, there is growing evidence that glucocorticoid receptor antagonists, such as mifepristone (RU486), may be useful in treating some cases of depression (Aisa et al., 2007; Flores et al., 2006).

1.4.4. New theories

Multiple studies have demonstrated the involvement of other neurotransmitter systems in the etiology and hence, in the subsequent treatment of depression (Charney, 2000), e.g. cytokines, neuropeptide neurotransmissors, such as CRF, TRH, somatostatine or growth hormone releasing factor GHRF.

The ultimate goal of these new perspectives of treatment is to reach higher therapeutic response with lesser side effects.

Dysfunction in glutamatergic transmission

A number of studies have reflected certain alterations in glutamate levels in affective disorders, such as reduction on glutamate levels in the anterior cingulate cortex (Auer et al., 2000).

Abnormalities in L-glutamic acid and γ-aminobutyric acid signal transmission have been postulated to play a role in depression (Choudary et al., 2005). A significant down regulation of two key members of the glutamate transporter protein family in astroglia and a decrease of L-glutamate-ammonia ligase (enzyme that converts glutamate to nontoxic glutamine) could elevate levels of extracellular glutamate considerably, which is potencially neurotoxic and can affect the efficiency of glutamate signaling (Choudary et al., 2005).

Studies based on the deleterious effects of stress (see Introduction 1.4.3.) suggest that an inhibition of glutamate release coud serve as a valid therapeutic approach. According to the results of these studies, glucocorticoids and stress have damaging effects on hippocampal cell morphology and survival, mediated via an excitatory amino acid mechanism (Lowy et al., 1993), and there is a direct evidence that stress increases the neuronal extracellular release of excitatory amino acids (e.g. aspartate, glutamate) in a regionally selective manner (Moghaddam et al., 2002). In addition to these latter results, certain dysfunction in NMDA receptors expression associated to depression was reported. In fact, a reduction in glycine affinity to the glutamate NMDA receptors occured in frontal cortex of human suicide victims. These data would support the hypothesis that glutamatergic dysfunction is involved in the psychopathology underlying suicide and, potentially in human major depression (Nowak et al., 1995).

Results of post-mortem studies (e.g. Meador-Woodruff et al., 2001) revealed differences in the expression of different NMDA and AMPA receptor subunits mRNA in the striatum from bipolar and major depressive disorders samples, although these results were isolated and minimal. Moreover, a reduction in the expression of GluR1 mRNA, an AMPA receptor subunit, was found in bipolar disorder (Meador-Woodruff et al., 2001).

In experimental studies performed on animals, an exposition to stress evokes an increase in the mRNA levels of NR1 and NR2 subunits of NMDA receptors and GluR1 subunit of

AMPA receptors in the rat hippocampus, as well as the expression of NR1 and GluR1 subunits in the ventral tegmental area (Schwendt et al., 2000; Fitzgerald et al., 1996).

Synaptic plasticity

Recent studies have begun to characterize adaptations of neuronal morphology and survival at the cellular level, and the intracellular signal transduction cascades at the molecular level, that underlie the response to antidepressant treatments. Adaptations at the cellular and molecular levels in response to stress and antidepressant treatment could represent a form of neural plasticity that contributes to the pathophysiology and treatment of depression. Depression itself could result from an inability to make the appropriate adaptive responses, such as neuronal atrophy and loss of plasticity, to stress or other aversive stimuli (Duman et al., 1999). Antidepressant medications may act by correcting this dysfunction or by themselves directly inducing by themselves the appropriate adaptive responses involving activation of members of the neurotrophin family of peptides. Chronic antidepressant administration increases the expression of transcription factor CREB (a single component of a complex of promoter-bound enhancing factors) in rat hippocampus. Up regulation of CREB mRNA was observed after chronic, but not acute, administration, consistent with the time course of the therapeutic action of antidepressant. The function of CREB is mediated by activation of the cAMP cascade. In this way, CREB could be activated by 5-HT and NA receptors that directly stimulate cAMP production (e.g., 5-$HT_{4\text{-}6\text{-}7}$ or β-adrenergic receptors). In addition, the function of CREB may be regulated by other 5-HT or NA receptors (e.g., 5-$HT_{2A,C}$ and α1-adrenergic receptors) (Nibuya et al., 1999). Moreover, when studying the effects of over-expression of CREB in rat dentate gyrus in the forced swimming test or learned helplessness paradigm (two animal models widely accepted for the study of depression), it was found that CREB acted as an antidepressant treatment (Chen et al., 2001a). In post-mortem studies on depressive patients, a reduction of CREB in the temporal cortex, reversed by antidepressant treatment, was observed (Dowlatshahi et al., 1998).

Another factor involved in the neuroplasticity and survival at the cellular level is BDNF. It is a commonly held idea that a major mechanism by which extracellular stimuli, such as neurotrophins and neuronal activity, regulate the survival of specific populations of developing neurons is through the activation or repression of gene transcription. BDNF promotes granule cell survival by activation of MAP kinase cascade culminating in CREB

phosphorylation and direct inhibition of the pro-apoptotic *bcl-2* family member BAD and by transcription-dependent mechanisms involving CREB, such as induction of expression of the anti-apoptotic *bcl-2* protein (Finkbeiner et al., 2000). Recent studies demonstrate that chronic antidepressant treatment is capable to increase the intensity of Bcl-2 immunostaining in rat hippocampus (Xu et al., 2003). Moreover, BNDF induce similar effects to those induced by antidepressants in the forced swim test or learned helplessness paradigm (Siuciak et al., 1997). In post-mortem studies on depressive patients and at similarity to the results described involving CREB, an increase of BNDF levels in the dentate gyrus, hilus and supragranular regions in treated patients was observed, whereas low serum levels of BNDF were detected in untreated patients (Karege et al., 2002; Chen et al., 2001b).

Furthermore, it was found that acute stress suppresses the neurogenesis under the actions of adrenal steroids (glucocorticoids) and NMDA receptor-mediated excitatory input (Gould et al., 1998). Likewise, it was observed that glutamate, acting via NMDA receptors, inhibits the neurogenesis, whereas NMDA receptor antagonists, e.g. MK-801, exhibit the opposite effect (Gould et al., 1994). Moreover, mood stabilizers, such as lithium, and antidepressants exert neurotrophic effects in dentate nucleus and may therefore be of use in the long-term treatment of other neuropsychiatric disorders (Chen et al., 2000; Malberg et al., 2000). Taken all these date, it could be hypothesized that the "therapeutic lag" until the antidepressant treatment is effective, may be due to the time it takes for newly born dentate gyrus neurons to migrate and differentiate, and to extend their neurites and become fully functionally integrated into the existing brain circuitry (Jacobs et al., 2002).

1.5. Antidepressant treatment

Nowadays, the pharmacological treatment of depression is based on restoration of NA and 5-HT levels, deficient in the disease process, according to the monoamine hypothesis of depression (Schildkraut et al., 1965). Currently available treatments for depression include tricyclic antidepressants (TCA), selective serotonin reuptake inhibitors (SSRIs), serotonin/norepinephrine reuptake inhibitors (SNRIs), various atypical antidepressants, and electroconvulsive therapy. Monoamine oxidase inhibitors (MAOI), discovered by chance in 1950s, also have played a theoretical and practical role in therapeutics (Tamminga et al., 2002). Although these treatments are effective, a significant number of patients do not

respond or achieve sustained remission despite aggressive management. Treatment is often accompanied by limiting side effects (Holtzheimer and Nemeroff, 2006; Tamminga et al., 2002) such as TCA related cardiotoxicity or MAOI related hypertensive crisis. However, the introduction of the SSRIs has improved the safety and tolerability of antidepressant medications, mainly due to the absence of adverse anticholinergic effects (Blier, 2002). However, other side effect such as nausea, anxiety, insomnia, anorexia, migraine or sexual dysfunction persist. One of the main objectives in the search for new medications to reduce the lag of the onset of the therapeutic activity of antidepressants (Blier, 2002).

The main groups of antidepressant treatment, accompanied by several examples are resumed onwards:

- **Tricyclic antidepressants**

Amitriptiline, nortriptiline, protriptiline, imipramine, desipramine, clomipramine, trimipramine and doxepine.

- **Selective 5-HT reuptake inhibitors (SSRIs)**

Fluoxetine, citalopram, paroxetine, sertraline and fluvoxamine.

- **Serotonin norepinephrine reuptake inhibitors (SNRIs)**

Venlafaxine, milnacipram and duloxetine.

- **Serotonergic and/or adrenergic receptor antagonists**

Trazodone: 5-HT_{2A} antagonist and 5-HT reuptake inhibitor.
Nefazodone: 5-HT_{2A} and α-adrenergic antagonist.
Mirtazapine: 5-HT_{2A} and central presynaptic α_2-adrenergic antagonist.
Mianserine: α_1 and α_2 antagonist.

- **Selective dopamine and norepinephrine reuptake inhibitor**

Bupropion.

- **Monoamine oxidase inhibitors (MAOIs)**

Irreversible inhibitors: *iproniazide, fenelzine and tranilcipromine.*

Reversible MAO A inhibitors: *moclobemide and blefoxatone*.

1.6. Novel targets for antidepressant treatment

Advances in the neurobiology of depression have pointed to a number of novel targets for antidepressant treatment. Based on an improved understanding of the neurobiology of depression, several novel pharmacologic and nonpharmacologic interventions are being developed. Pharmacologic developments include CRF antagonists such as R121919 (Zobel et al., 2000); glucocorticoid receptor antagonists; NK1 receptor antagonists (Stout et al., 2001); postsynaptic NMDA glutamate receptor antagonists and postsynaptic AMPA receptor amplifiers; transdermal selegiline (MAO-B inhibitor); so-called "triple" reuptake inhibitors (Holtzheimer and Nemeroff, 2006); inductors of neuroprotective/neurogenic effects such as neurotrophines (BDNF) or inductors of antiapoptotic Bcl-2 gene expression. Nonpharmacologic advances have largely involved focal brain stimulation techniques including vagus nerve stimulation, transcranial magnetic stimulation, magnetic seizure therapy, and deep brain stimulation. Data on these treatments are preliminary yet, and more studies are needed to clarify their potential clinical benefit. However, it is clear that further study of the neurobiology of depression will continue to provide a rationale for developing innovative targets for antidepressant therapies (Holtzheimer and Nemeroff, 2006).

2. THE GABAERGIC SYSTEM

γ-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the adult mammalian brain, where it is widely distributed (Brambilla et al., 2003). It is involved in the control of inhibitory-excitatory balance (Grifa et al., 1998). GABA was first identificated and localized in vertebrated by two different groups (Roberts and Frankel et al., 1950). Other aminoacids have gained the importance as possible neurotransmitters in adult mammalian central nervous system. There are two main groups of aminoacid neurotransmitters:

- *excitatory aminoacids*: glutamate, aspartate, cysteine, homocysteine
- *inhibitory aminoacids*: GABA, glycine, taurine, β-alanine

The potential involvement of a GABAergic dysfunction in mood disorders was first proposed in 1980 by Emrich et al, based on the efficacy of valproate in the treatment of

bipolar patients. It was proposed that valproate, through the enhancement of GABA brain concentration, might compensate for a potential GABAergic deficiency, and formulated the GABA hypothesis of mood disorders. After Emrich's hypothesis, several animal and human studies have evaluated the potential role of GABAergic abnormalities in the pathophysiology of mood disorders (Brambilla et al., 2003). Dysfunction of GABA-mediated synaptic transmission in the CNS is therefore believed to underlie various nervous system disorders. For example, hypoactivity of the GABA system has been linked to epilepsy, Alzheimer disease, memory, spasticity, anxiety, stress, sleep disorders, depression, addiction, and pain. On the contrary, hyperactivity of the GABAergic system has been associated with schizophrenia (Bettler et al., 2004).

Preclinical studies have suggested that GABA levels may be decreased in animal models of depression, and clinical studies reported low plasma and cerebrospinal fluid GABA levels in mood disorder patients. Also, antidepressants, mood stabilizers, electroconvulsive therapy, and GABA agonists have been shown to reverse a depression-like behaviour in animal models and to be effective in the treatment of unipolar and bipolar patients by increasing brain GABAergic activity. The hypothesis of reduced GABAergic activity in mood disorders may complement the monoaminergic theory of depression and it can be proposed that the balance between multiple neurotransmitter systems may be altered in these disorders (Brambilla et al., 2003).

GABA exerts its effects on ionic $GABA_A$ or $GABA_C$ receptors that mediate a fast inhibition via Cl^- channels, or on metabotropic $GABA_B$ receptors coupled with G-protein that activates second messenger systems and Ca^{2+} and K^+ ion channels, which mediate a late inhibition.

2.1. GABA synthesis, metabolisms and reuptake

As shown in Figure 3, in the GABAergic terminals, GABA is synthesized from glutamate. The whole synthesizing mechanism starts when α-cetoglutarate formed and transaminated via reaction catalyzed by GABA-α-oxoglutarate transaminase (GABA-T) in order to form L-glutamic acid which is then decarboxylated in an enzymatic reaction mediated by glutamic acid decarboxylase (GAD), an enzyme consistently implicated in mood disorders (Choudary et al., 2005) and a limiting factor of GABA biosynthesis. GAD is

expressed only in GABAergic neurons and several peripheral tissues, e.g. retina, pancreas (Erlander and Tobin, 1991). The existence of GAD provide an important marker for GABA neurons identification. Two forms of GAD of different molecular weight, peptide sequence and subcellular localization have been discovered so far: GAD_{67}, distributed in neurosome, and GAD_{65}, localized in presynaptic terminals (Kaufman et al., 1991). GABA is stored in presynaptic vesicles and liberated by Ca^{2+} dependent exocytosis from neural terminals after presynaptic membrane depolarization.

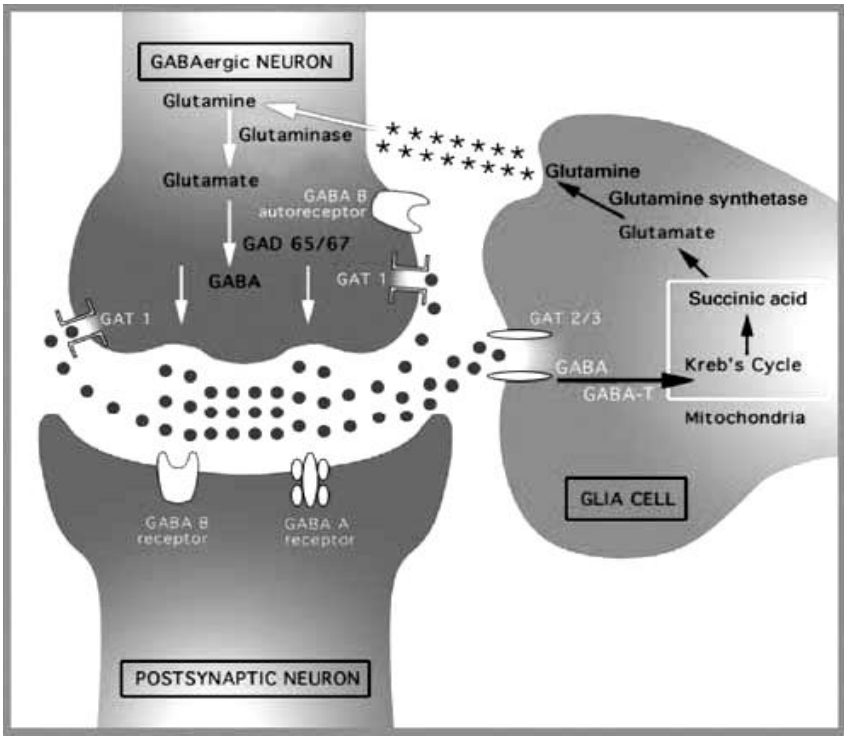

Figure 3. GABA synthesis, metabolism and uptake in human brain. Glutamate is the precursor of GABA in GABAergic terminals. Glutamate comes from two different sources, Kreb's cycle in glia cells and glutamine in nerve terminals. Then the enzyme glutamic acid decarboxylase (GAD) forms GABA from glutamate. After being released into the synapses, GABA is inactivated by reuptake mediated by GABA transporters (GATs) into presynaptic terminals or into glia cells where it is metabolized by GABA transaminase (GABA-T).

After being released into the synapses, GABA is inactivated by reuptake into presynaptic terminals or into glia cells mediated by means of GABA transporters (GATs). Based in immunocytochemical experiments employing polyclonal antibodies, GABA transporters have been localized in many cerebral regions (Gadea et al., 2001; Radian et al., 1990) such as the cerebral cortex, hippocampus, thalamus, basal ganglia, cerebellum,

hypothalamus, and brainstem, in which it represents about one-third of the synapses (Brambilla et al., 2003). Specifically, at the present time, four complementary DNAs (cDNAs) encoding highly homologous GATs proteins have been cloned (GAT-1, GAT-2, GAT-3, and BGT-1). GAT-1 is considered to be a neuronal transporter, GAT-2 and GAT-3 are believed to be glial transporters, whereas the role of BGT-1 in brain GABA uptake is unknown. Precisely, GAT-1 is the most frecuently expressed GAT in the CNS and it is mainly located into presynaptic axon terminal and into few astrocytic processes. GAT-2 is primarily present in the leptomeninges and in ependymal and choroid plexus cells and, to a minor extent, in neuronal and non-neuronal elements. GAT-3 is located exclusively to distal astrocytic processes, although a neuronal localization has been reported in some brain regions such as the retina. GATs are regulated by several factors including GABA itself, BDNF and hormones. The different response of GATs to the composition of extracellular environment, the different regulation of their activity and/or expression, and the possibility of reversing the direction of GABA transport, confer to the GABA transport system considerable flexibility for the fine regulation of GABA levels under physiological and pathological conditions.

GABA that is taken up by astrocytes is not immediately available for synaptic transmission, because it is metabolized to succinic semialdehyde (SSA) by GABA-transaminase (GABA-T), which uses pyridoxal phosphate as a cofactor. GABAergic reuptake by the presynaptic membrane transporters is almost 4-5 times higher as compared with reuptake by astrocytes, where the GABA-T activity is predominant. This may explain the reason why once GABA is taken up, it is recycled directly into synaptic vesicles and utilized in new transmission cycle in preference to be catabolized by GABA-T (Bradford, 1995). Then, succinic semialdehyde is oxidized either by succinic semialdehyde dehydrogenase (SSA-DH) to succinic acid (SA), which re-enters the Kreb's cycle and then is transformed into glutamate, or by aldehyde reductase to γ-hydroxybutyrate.

Glutamate cannot be converted into GABA in astrocytes due to the absence of GAD, and it is transformed by glutamine synthetase into glutamine, which is then transferred to axon terminals by specific transporters. In nerve terminals, glutamine is then converted into glutamate by the enzyme glutaminase, and, finally, GAD forms GABA from glutamate closing the cycle (Figure 3). On the contrary, GABA that is taken up by neuronal transporters is readily available for further release, because it either undergoes the same transformation as in astrocytes (with the notable difference that nerve endings contain GAD and can

resynthesize GABA) or it is recycled directly into synaptic vesicles. Glutamine synthetase is present only in glia, whereas GABA-T and SSA-DH are found in neuronal and glial mitochondria.

2.2. GABA receptors

2.2.1. $GABA_A$ receptors

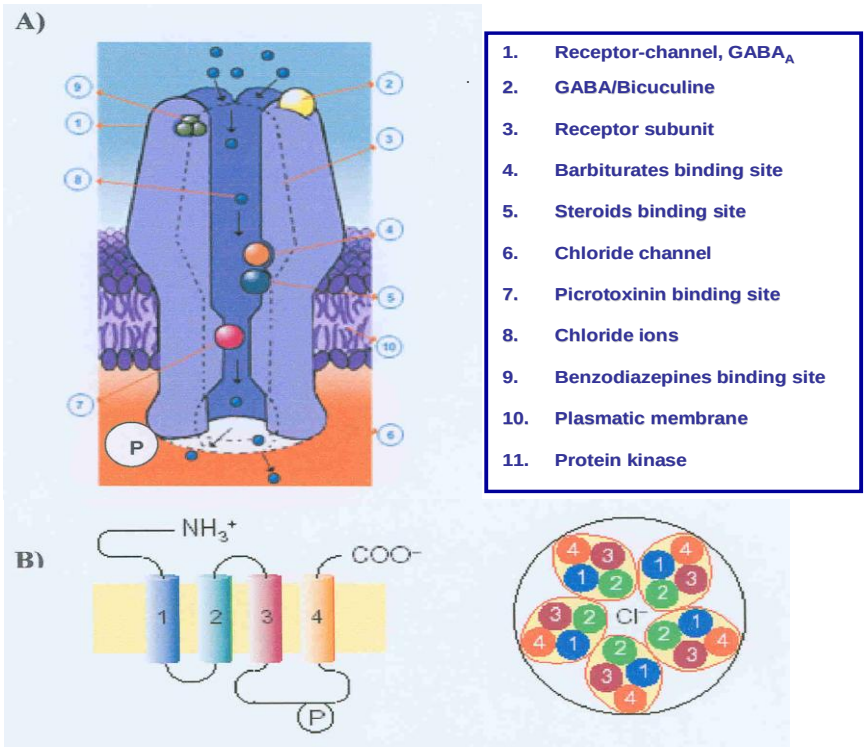

Figure 4. $GABA_A$ receptors. **(A)** The $GABA_A$ receptor is a Cl^- ionophore with ~5 Å diameter and modulatory binding sites for benzodiazepines, barbiturates and neurosteroids. GABA responses are blocked competitively by bicuculline and noncompetitively by picrotoxinin; they are modulated intracellularly by protein kinases, such as protein kinases A and C. The vertebrate $GABA_A$ receptor complex is built from five subunits belonging to different families (α1–6, β1–4, γ1–4, δ, ε and π). **(B)** Each subunit comprises four transmembrane domains (TM1–TM4). The large intracellular loop between TM3 and TM4 contains consensus sites for phosphorylation by protein kinases (P). The amphiphilic TM2 provides the lining of the Cl^- pore intrinsic to the pentameric structure. The most abundant $GABA_A$ receptor in the brain is the α1β2γ2 isoform.

$GABA_A$ receptors are ionotropic and mostly postsynaptic receptors mainly located at the apical dendrite of the neurons, causing the fast inhibitory postsynaptic potential (IPSP). $GABA_A$ receptors belong to the *ligand-gated ion chanel* receptor family (Cys-loop LGIC) together with nicotinic, glutamaergic, glycine and 5-HT_3 receptors. They are hetero-

oligomeric (a pentameric complex) membrane proteins organized in a channel, composed of five subunits belonging to several different classes with multiple variants (α1–6; β1–4, γ1–4, δ, ε, θ, π, and ρ1-3). Each subunit has a large extracellular domain – N terminus, four hydrophobic transmembrane domains (TM1-TM4) – an intracellular loop containing protein kinase A, protein kinase C, and tyrosine kinase phosphorylation sites, and a short C terminus – , and a large cytoplasmatic domain. Between TM3 and TM4, there is an extracellular domain of specific dimension and sequence for each subunit, where intracellular regulatory mechanisms sensitive to diverse kinases phosphorylation, are exerted. $GABA_A$ receptor usually contains α, β, and γ subunits with variable combinations, which may be relevant to pharmacological differences in responses to drugs and that may modulate receptor activity. In the mammalian brain, α1, β2, γ2 is the major $GABA_A$ receptor subunit conformation (see Figure 4). During neurotransmission, GABA acts postsynaptically through allosteric interaction with $GABA_A$ receptors and allows the chloride (Cl^-) ion channel to open, increasing the Cl^- conductance. Once $GABA_A$ receptors are activated, hyperpolarization of the neuronal membrane is established, reducing the cell excitability and leading to the inhibitory actions of GABA. However, in the presence of chronic GABA administration, Cl^- currents gradually decrease. It has been shown that phosphorylation and dephosphorylation processes might regulate $GABA_A$ receptor function. For instance, it has been reported that in specific brain cells, both protein kinase A (PKA) and C (PKC) modulate the minimal inhibitory postsynaptic currents. Moreover, it has been reported that zinc, a divalent cation that is known to regulate synaptic excitation, inhibits GABA-mediated responses through an interaction with histidine residues on the $GABA_A$ receptor complex. Probably, the sensitivity of $GABA_A$ receptors to zinc may be enhanced or reduced in the presence of the subunit α6 or γ2, respectively. Furthermore, growing evidence has demonstrated that GABAergic transmission can be potentiated via neurosteroids by interaction with $GABA_A$ receptors, particularly the α and δ subunits (Brambilla et al., 2003).

This high heterogeneity of $GABA_A$ receptors in different cerebral regions increase the possibility to develop selective ligands for each subtype of the receptor . This perspective has been supported by multiple modulation sites localizated in different subtypes of $GABA_A$ receptors of which a limited number of specific ligand has been established.

Allosteric modulators

According to the above mentioned, the molecular complex of $GABA_A$ receptor is formed by a chloride ion channel, GABA binding sites, benzodiazepines (BDZs) recognition sites and other binding sites for different ligands, such as barbiturates, ethanol, anticonvulsants, neurosteroids, steroid anesthetics, and volatile general anesthetics. All these drugs act as allosteric agents, leading to increased GABA affinity and increased frequency of chloride channel opening (Brambilla et al., 2003). These compounds can also modify the afinity of the rest of elements for their appropriate binding sites (Olsen et al., 1997; Sarter and Bruno, 1994). Specifically, BZDs bind to the subunit α and increase the affinity of the receptor for GABA (Brambilla et al., 2003). This indirect effect on the GABA receptor contribute to the low BDZ toxicity and large clinical application scale (from anxiety disorders to epilepsy). In addition, once the saturation levels of BDZ are achieved, they practically lose the therapeutic effect (Zorumski and Isemberg, 1991), in contrast to barbiturates that exert a dual action (Skolnick et al., 1981). Barbiturates, at low concentrations increase GABA afinity and time of chloride channel opening, whereas at higher concentrations lead to direct chloride channel opening even though of GABA is absence from the media.

BDZs produce most, if not all, of their numerous effects on the CNS primarily by increasing the function of those chemical synapses that use GABA as transmitter (Hunkeler et al., 1981). BDZ binding site occur in the extracellular N-terminus part of $GABA_A$ receptor. Classic BDZs, such as diazepam and midazolam, increase the function of $GABA_A$ receptors, induce muscular relaxation and sedation and have anticonvulsive and anxiolytic effects, although they can also induce amnesia (Rudolph et al., 2004). Radioligand binding studies demonstrated two types of BDZ receptors, I and II (Pritchett et al., 1989) based on different affinity for CL 218872 (a partial agonist of triazolopyridazine structure), also named as BZ_1 and BZ_2 (Lippa et al., 1981) when affinity is determined with β-carboline.

Diazepam has been reported to increase the brain peak GABA-evoked current by accelerating GABA association to its receptors, and to enhance CSF GABA levels in humans. Human plasma GABA levels have been shown to be reduced by diazepam and lorazepam administration. Functional studies reported that $GABA_A$ receptors may be involved in BDZ effects on cerebral metabolism and in BDZ tolerance in humans. Also, the reported efficacy of BDZs in treating acute mood disorder patients is consistent with the hypothesis of a

GABAergic deficit in mood disorders. Clonazepam and lorazepam have indeed been suggested to be useful in manic patients. Clonazepam and alprazolam have also been reported to be efficacious in treating depression in bipolar and unipolar patients. Alprazolam should generally be avoided in the treatment of manic states in bipolar patients, as cases of alprazolam-induced mania have been reported (Bormann and Leader, 2000).

Other important group of benzodiazepine ligands is formed by inverse agonists (β-carboline derivates) that decrease GABA induced ion conductance (Sarter and Bruno, 1994; Sarter and Bruno, 1988) and cause convulsive and anxiety effects. Nowadays, partial BDZ agonists (imidazenil) have been developed, which maintain the anxiolytic effects without side effects such as sedative effects, tolerance and addiction (Kleven, 1999; Zanotti et al., 1996; Haefely, 1990). Selective antagonists such as flumazenil do not influence GABA induced chloride flow but antagonize agonist or inverse agonist action, since they bind to the BDZ recognition binding site of $GABA_A$ receptor. Flumazenil is benefical in anaesthesiology to revert BDZ induced sedation, although recent studies have been demonstrated the possible efficacy of flumazenil in pathophysiology of hepatic encephalopathy (Amaral, 1999; Barbaro, 1998).

Regarding the binding site in $GABA_A$ receptors for steroid compounds, a number of neurosteroids such as alopregnolone are suggested to be potent positive modulators. Alphaxolone, a steroid with potent anesthetic action, increase $GABA_A$ receptor activation under the action of GABA (Harrison et al., 1984). Moreover, other neurosteroids, e.g. pregnanolone sulphate or dehydroepiandrosterone (DHEA) also act as negative modulators (Majewska and Schwartz, 1987). Recent studies demonstrate that cortisol is a potent bi-directional $GABA_A$ receptor modulator, positive at low concentrations but negative at high concentrations. In the same way, corticosterone, a noncompetitive antagonist of the neurosteroid $GABA_A$ receptor binding site, is one of the most potent agents acting on the latter binding site (Sieghart, 1995).

Agonists and antagonists

Heterogeneity of $GABA_A$ receptors in different cerebral regions increase the possibility to develop selective ligands for each subtype of the receptor. Muscimol, an analog of GABA isolated from the mushroom *Amanita muscaria,* is considered to be one of the most selective

and potent agonists so far. Bicuculline is the prototype of $GABA_A$ receptor competitive antagonist and lead to increased GABA affinity and increased frequency of chloride channel opening. Picrotoxine and t-butylbicyclophosphotothionate (TBPS) are competitive antagonist that bind to a different site of the receptor than GABA. Both classes of antagonists produce convulsions when administrated to experimental animals (Steven, 1995). The therapeutic use of both agonists and antagonists is associated with severe side effecs. Total agonists can induce the desensibilization of target receptors leading to the tolerance and consequent symptoms, whereas antagonists might produce anxiety and convulsions. Therefore, investigation on possible therapeutic molecules is being focused on partial $GABA_A$ receptor agonists.

2.2.2. $GABA_B$ receptors

$GABA_B$ receptors were first described by Hill and Bowery in 1981 as GABA receptors insensitive to bicuculline but sensitive to baclofen. They are not linked to the BDZs recognition sites and their structures are less well characterized than $GABA_A$ receptors (Brambilla et al., 2003). In contrast to ionotropic $GABA_A$ and $GABA_C$ receptors, these are metabotropic receptors coupled to heterotrimeric G proteins of the G_i- and G_o-type, probably in a stochiometry of one receptor heterodimer to one G protein (see Figure 5). Upon activation of the G protein, the βγ complex basically supress the neuronal calcium conductance (inhibits Ca^{2+} channels) and activates K^+ channels, whereas the α subunit influences the level of the second messenger cyclic adenosine monophosphate (cAMP) by regulating adenylate cyclase activities.

The major effect of $GABA_B$ receptors in presynaptic nerve terminals is the voltage-dependent inhibition of Ca^{2+} channels, resulting in presynaptic inhibition of elicited neurotransmitter release. Presynaptic $GABA_B$ receptors regulating GABA release are termed autoreceptors, whereas $GABA_B$ receptors controlling the release of other neurotransmitters are called heteroreceptors. The βγ-mediated inhibition of the Ca^{2+} channels is relieved by strong depolarization, which may result in a differential frequency-dependent modulation of action potential trains.

On the postsynaptic side, $GABA_B$ receptors induce a slow inhibitory postsynaptic current (sIPSC) mediated by G-protein-activated inwardly rectifying K^+ channels. Upon activation by the G protein βγ complex, these channels allow an increased efflux of K^+ resulting in a slow hyperpolarization of the postsynaptic membrane (Kornau, 2006). In summary, presynaptic $GABA_B$ receptors primarily regulate transmitter release by the inhibition of Ca^{2+} channels, whereas postsynaptic $GABA_B$ receptors produces an increase in membrane K^+ conductance and associated neuronal hyperpolarization (Brambilla et al., 2003). However, $GABA_B$-receptor-mediated inhibition of postsynaptic Ca^{2+} channels and the activation of presynaptic K^+ currents have also been observed (Kornau, 2006).

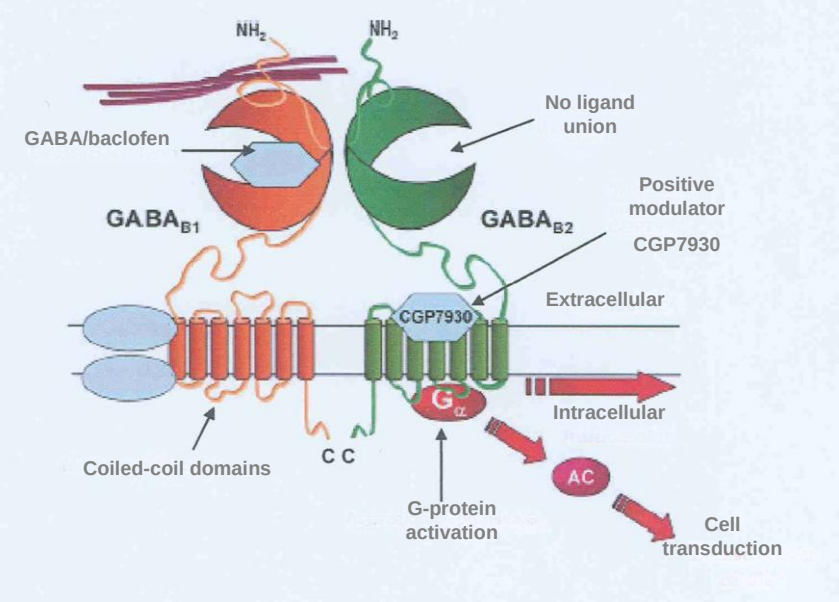

Figure 5. Metabotropic $GABA_B$ receptors are heterodimers consisting of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits. The heterodimerization is facilitated by coiled-coil domains, all the agonists and antagonist known up to date bind to $GABA_{B(1)}$ subunit, whereas the activation of the receptor is mediated by $GABA_{B(2)}$ subunit. Recently, the union of positive allosteric modulators such as CGP7930 occur in the transmembrane domain of the $GABA_{B(2)}$ subunit.

The $GABA_B$ receptor belongs to Class-III GPCRs (G-protein-coupled receptors). The GPCRs form a large family of membrane proteins responsible for transduction of various external signals into intracellular responses through heterotrimeric G-proteins. Five main classes have been described based on their structural features. Knockout studies demonstrate that $GABA_{B(1)}$ is an essential component of pre- and postsynaptic $GABA_B$ receptors in the CNS which rules out the presence of functional homomeric $GABA_{B(2)}$ receptors and is consistent with the proposal that the $GABA_{B(1)}$ subunit is solely responsible for binding GABA (Bettler et al., 2004), making the $GABA_B$ receptor an obligatory heterodimeric

receptor. While the $GABA_{B1}$-ECD (extracellular domain) has been shown to bind GABA, the $GABA_{B2}$-HD (heptahelical domain) is responsible for coupling with G-proteins (Binet et al., 2004).

$GABA_{B(1a)}$ and $GABA_{B(1b)}$ subunits form the basis of two proteins highly expressed in the CNS. The isoforms may differ in their pre- and postsynaptic distribution. Knockout studies further indicate that the $GABA_{B(1)}$ subunit is an essential requirement for $GABA_B$ receptor function in the peripheral nervous system (PNS) and the enteric nervous system (Bettler et al., 2004).

Allosteric modulators

Recently, ligands acting directly on class-III GPCR HDs have been identified. For example, the action of positive and negative allosteric modulators of mGluR5 (metabotropic glutamate receptor 5) has been characterized (Binet et al., 2004).

The allosteric modulators are able to distinguish between ativated and inactivated receptor. They lead to increased endogenous GABA activity, whereas agonists activate each $GABA_B$ receptor, independently of the synaptic activity. The first synthetic compounds with $GABA_B$ allosteric activity but structurally different from $GABA_B$ agonists (Urwyler et al., 2003; Urwyler et al., 2001), such as CGP7930, CGP13501, GS39783 increase considerably the responses to $GABA_B$ agonists. CGP7930 is a positive allosteric modulator of the $GABA_B$ receptor, since it increases the maximal effect and the potency of GABA in assays measuring both the GTP[^{35}S] binding on G-proteins and inositol phosphate production. Thus CGP7930 binds to the HD of the $GABA_B$ receptor. Moreover, because of the ability of the $GABA_{B2}$ subunit to couple with G-proteins, it was observed that CGP7930 acts directly on the $GABA_{B2}$-HD. Apart from the fact that the positive allosteric modulator CGP7930 is an excellent pharmacological tool to explore the molecular mechanisms of HD activation, its original binding at a site distinct from that of GABA and the large implication of the $GABA_B$ receptor in physiology and pathology suggest that such compounds are promising drugs for therapeutical application (Binet et al., 2004).

Agonists and antagonists

In 1962 the chemist Heinrich Keberle synthesized a lipophilic derivative of GABA, β-*p*-chlorophenyl-GABA, baclofen, which enters the CNS to a small extent sufficient to exert valuable muscle relaxant and analgesic properties and is still widely prescribed for the treatment of spasticity in patients suffering from multiple sclerosis or hemi- and tetraplegia (Froestl et al., 2003). However, its numerous disfavourable side effects, such as sedation, tolerance or motor disturbances are limiting for its use in treatment of many diseases.

At the end of eighties, the first selective antagonists for $GABA_B$ receptor such as faclofen, saclofen and 2-hydroxysaclofen were described (Kerr et al., 1992). In 1984 chemists at the Central Research Laboratories of Ciba–Geigy in Manchester discovered a novel class of $GABA_B$ receptor ligands, phosphinic acid analogues of GABA, e.g. CGP27492 which showed a very high affinity towards $GABA_B$ receptors exceeding significantly the affinities of both GABA and baclofen. CGP55845A or CGP56433A are other more selective and potent antagonists developed in the eighties by Froestl *et al* (2003). Nowadays, a second generation of these antagonists are still being investigated (Bowery, 2002). Moreover, CGP36742 is being examinated in second phase of clinical trials for its pro-cognitive activities in Alzheimer disease treatment (Froestl et al., 2004).

2.2.3. $GABA_C$ receptors

Although both $GABA_C$ and $GABA_A$ receptors are ionotropic receptors, $GABA_C$ receptors are structurally distinct from $GABA_A$ receptors. Fully functional $GABA_A$ receptors require heterooligomeric formation of α-, β- and γ-subunits (Kornau et al., 2006), while $GABA_C$ receptors can assemble as homooligomers (formed by ρ1 ρ2 ρ3) (Orgurusu et al., 1999; Enz et al., 1998) or pseudohomooligomers (with combinations ρ1 ρ2 or ρ2 ρ3). To date, 5 different ρ-subunits (ρ1–ρ5) have been cloned from several mammalian and vertebrate species, more specifically from human, rat, mouse, chicken and perch retina (Chebib et al., 2000), of which only 2 (ρ1 and ρ2) have been cloned from humans, and 3 in rats (ρ1 ρ2 ρ3). The ρ-subunits share only 30–38% amino acid sequence identity with the $GABA_A$ receptor subunits and they mediate robust bicuculline-insensitive GABA responses in heterologous expression systems. There is no evidence so far from heterologous expression systems that the

ρ-subunits coassemble with the $GABA_A$ receptor α-, β- and γ-subunits, or with the glycine receptor β-subunit.

In the rat retina, $GABA_C$ receptors are probably pseudohomooligomers composed of ρ1- and ρ2-subunits; the ρ1ρ2 receptors display characteristic activation and inactivation properties that differ from those of either form of homooligomer. Because the ρ1-subunit is expressed predominantly in the retina, $GABA_C$ receptors in other CNS regions are probably ρ2 homooligomers. $GABA_C$ receptors show a distinct cellular and subcellular localization. Although $GABA_A$ receptors are present in all CNS regions, $GABA_C$ receptors are highly enriched in the vertebrate retina. Using a polyclonal antibody, the ρ-subunits have been localized to the axon terminals and dendrites of bipolar cells (Kornau, 2006), although they have also been observed in thalamus (Hinton et al., 2003), hippocampus (Rozzo et al., 2002; Enz et al., 1995), pituitary (Boue-Grabot et al., 2000) and intestine (Jansen et al., 2000). Synaptic $GABA_C$ receptors comprising ρ-subunits are clustered into hot spots, but $GABA_C$ and $GABA_A$ receptor subunits do not colocalize in the same hot spots to form hybrid receptors. Moreover, ρ-subunits and glycine receptor subunits are also clustered at different synapses.

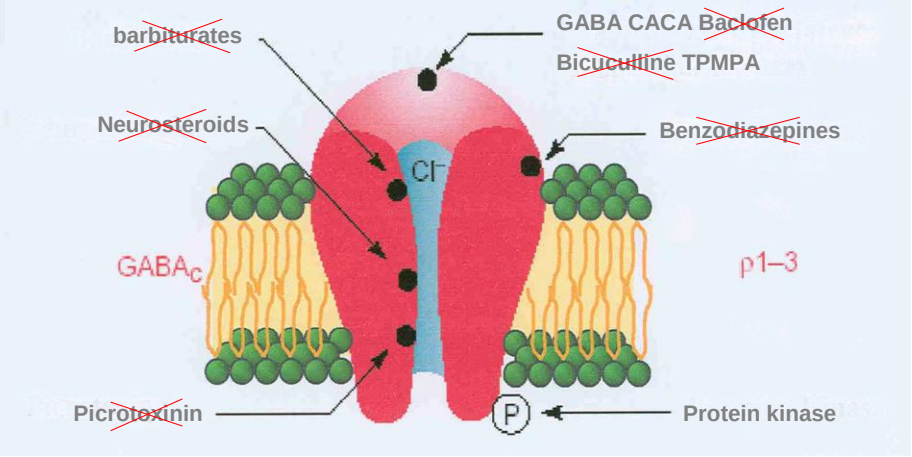

Figure 6. Ionotropic $GABA_C$ receptor forms a chloride channel and might be selectively activated by CACA and competitively blocked by TPMPA or noncompetitively blocked by picrotoxinin. Responses mediated by GABA might be inactivated by PKC phosphorylation.

The intracellular anchoring of $GABA_C$ receptors is also distinct from that of $GABA_A$ receptors. Colocalizaton of $GABA_C$ receptors and microtubule-associated protein (MAP-1B)

has been shown at postsynaptic sites on bipolar cell terminals, which indicates that $GABA_C$ receptors are linked to the cytoskeleton via MAP-1B. This cytoskeletal protein specifically interacts with the ρ1-subunit but not with the $GABA_A$ receptor subunits. For $GABA_A$ receptors, a new cellular protein, $GABA_A$-receptor-associated protein (GABARAP), has been identified, which interacts with the γ2-subunit and colocalizes with $GABA_A$ receptors on cortical neurones. These mechanisms might allow ionotropic $GABA_A$ and $GABA_C$ receptors to be differentially expressed at inhibitory synapses.

$GABA_C$ receptors are also a pharmacologically distinct group. Whereas $GABA_A$ and $GABA_B$ receptors are defined by their respective sensitivities to bicuculline and baclofen, $GABA_C$ receptors do not respond to either drugs. Notably, CACA (*cis*-4-aminocrotonic acid) is a selective agonist for $GABA_C$ receptors but it is inactive at $GABA_A$ receptors, whereas the *trans*-enantiomer TACA shows no such preference. Furthermore, TPMPA [(1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid] has been identified as a potent and highly selective antagonist for $GABA_C$ receptors. $GABA_C$ receptors are insensitive to $GABA_A$-modulatory drugs such as benzodiazepines, barbiturates and neurosteroids (see Figure 6). The chloride channel blocker picrotoxinin is a strong antagonist at both $GABA_A$ and ρ1 homomeric $GABA_C$ receptors; however, ρ2 homooligomers and rat native $GABA_C$ receptors that are composed of ρ1ρ2 are rather insensitive to this compound.

Electrophysiological responses of native or recombinant $GABA_C$ receptors also differ markedly from those of $GABA_A$ receptors. $GABA_C$ receptors are about tenfold more sensitive to the physiological agonist; the Hill slopes are steeper for $GABA_C$ receptors, which probably reflects the presence of five ligand binding sites on $GABA_C$ receptors, whereas only two appear to be present on $GABA_A$ receptors. Both activation and inactivation time constants are very slow, albeit differing among ρ1, ρ2 and ρ1ρ2 $GABA_C$ receptors. These findings have been taken as further evidence for rat retinal $GABA_C$ receptors being ρ1ρ2 pseudohomooligomers. A remarkable and physiologically significant feature of $GABA_C$ receptors is their very weak desensitization, even at very high concentrations of agonist. The robust and sustained responses and the high agonist sensitivity make $GABA_C$ receptors ideally suited for mediating strong lateral inhibition in the vertebrate retina. At the molecular level, $GABA_C$ receptors display a very low single-channel conductance but rather long mean open times. As expected for ligand-gated chloride channels, both ionotropic GABA receptor types have a high Cl^- selectivity and a similar pore size (Kornau, 2006).

2.3. Psychopathological implications of GABAergic system nervous system disorders

Dysfunction of GABA-mediated synaptic transmission in the CNS is believed to underlie various nervous system disorders, e.g. epilepsy, Alzheimer disease, memory, spasticity, anxiety, stress, sleep disorders, depression, agression, addiction, pain or schizophrenia. The potential role of GABAergic abnormalities in the pathophysiology of depression and anxiety is described bellow.

2.3.1 Depression

Following the hypothesis proposed by Emrich et al (1980), numerous experimental studies in animals and humans have assessed the role of the GABAergic alterations in the pathophysiology of depression.

In animal models such as Porsolt forced swimming test, low GABA levels in nucleus accumbens, brainstem and frontal cortex have been detected (Borsini et al., 1987). Moreover, muscimol (a $GABA_A$ agonist) administration in these animals increases the immobility time (Poncelet et al., 1987), whereas picrotoxin, a $GABA_A$ antagonist, reduces the muscimol-induced reduction of the immobility. Furthermore, at similarity to muscimol administration, GABA administration in frontal neocortex and hippocampus of animals subjected to the learned helplessness paradigm reverts the depressive-like effects (Poncelet et al., 1987; Lloyd et al., 1983), whereas picrotoxin reduces the muscimol-induced effects. In addition, decreased $GABA_A$ receptors expression in frontal cortex, hippocampus and striatum of animals subjected to forced swimming test has been observed (Drugan et al., 1985).

In humans, low plasma and cerebrospinal fluid GABA levels were observed in unipolar (Gerner et al., 1984; Gold et al., 1980) and bipolar (Berrettini et al., 1983) depressive patients. Then, in a study on untreated depressive patients using magnetic resonance spectroscopy, (MRS) reduced GABA levels were detected (Sanacora et al., 1999).

Since depression is considered to be a highly hereditary disorder, researchers have focused on the link between GABA receptor genes and the disease. Thus, a different α_5, α_1 and α_3 subunits gene expression (GABRA5, GABRA1 and GABRA3 respectively) may be responsible for the structural abnormalities in GABA receptors by increasing the

susceptibility of the individual to the development of depression (Massat et al., 2001; Papadimitriou et al., 1998; De Bruyn et al., 1996).

Nowadays, GABAergic agents used in treatment of bipolar depression as mood stabilizers, e.g. gabapentin or valproate, increase both GABA levels by releasing to the synaptic cleft and GAD activity, or inhibit GABA-T action in different cerebral regions. Moreover, increased GABA levels in human plasma after valproate administration have also been observed (Shiah et al., 2000).

Besides the action of BDZs, another compounds have been revealed (Sanacora et al., 2002) to be concerned in the modulation of GABAergic transmission. Antidepressive compounds such as fluoxetine or citalopram are able to increase GABA levels in depressive patients (Sanacora et al., 2002).

2.3.2. Anxiety

Anxiety is described as an unpleasant emotional state of high energy that involves a complex combination of emotions that include fear, apprehension, and worry. It is often accompanied by physical sensations such as heart palpitations, nausea, chest pain, shortness of breath, or tension headache.

The DSM-IV (2000) classify anxiety into generalized anxiety disoder (GAD), obsessive-compulsive disorder (OCS), panic disorder (PD), social anxiety disorder (SAD), specific phobia (SP), post-traumaticstress disorder (PTSD), phobias that occurs comorbidly with above cited disorders, and drug-induced anxiety.

The efficiency of BDZs in the treatment of anxiety disorders supports the idea that the GABAergic system may play an important role in the development of anxiety (Shader and Greenblatt, 1995). GABA regulate the neuronal transmission approximately in one third of the neuronal synapses which are mediated, among other neurotransmitter-receptor systems, by adrenergic (Charney et al., 1995) or serotonergic (Eison and Eison, 1994) systems. These two neurotransmitter systems have been involved in the neurobiological basis of anxiety. Actually, it has been proposed that the anxiolytic actions of BDZs could be a consequence of the inhibition of 5-HT release in the limbic cerebral region.

It has also been suggested the existence of endogenous cerebral substances such as neuroactive steroids (acting as $GABA_A$ receptor positive allosteric modulators) that could intervene in the pathology of anxiety disorder (Gasior et al., 1999). Due to the high diversity of $GABA_A$ receptor subunits expression in CNS, it has been also proposed that the anxiety disorders could be a consequence of structural abnormalities of certain receptor subunits in distinct cerebral regions (Shader and Greenblatt, 1995).

Moreover, involvement of certain neuropeptides in the development of anxiety and regulation of anxiety mechanism cannot be excluded. Even more, various peptides with anxiogenic properties such as CRF or cholecystokinin have been described.

II. PLANS AND OBJECTIVES

Mood disorders are among the most prevalent, recurrent, and disabling of all illnesses (Costello et al., 2002). These facts make depression one of the main sanitary burdens. Actually, according to WHO, in 2020 depression is considered to become the second biggest lifelong disability worldwide. Hence, recent investigation has focused on the biological and neurochemical basis of depression in order to search for new therapeutical approaches in the treatment of the illness.

The monoaminergic hypothesis, one of the classical theories on the aethiology of depression, in its original form neither provides a full explanation for the therapeutic action of antidepressants nor clarifies the pathophysiology of depression (Hindmarch, 2001). Therefore, recent investigations have focused, among others, on long-term adaptive processes within the brain to postulate alternative hypotheses, e.g. molecular and cellular hypothesis of depression in which neural plasticity plays a major role, hypotheses based on altered responses to stress, or those dealing with a role of GABAergic dysfunction in the illness.

It has been previously described that exposure to early stressful adverse life events may increase vulnerability to psychopathology in adult life (Aisa et al., 2007; Swaab et al., 2005; Moghaddam, 2002). Depressive illness is presumed to result from an interaction between the effects of environmental stress and genetic/developmental predisposition (Swaab et al., 2005). Almost all environmental and genetic risk factors for depression appear to correlate with increased HPA-axis activity. On the other hand, when patients or animals are treated with antidepressants, electroconvulsive therapy, or when they show spontaneous remission of the illness, the HPA-axis function returns to normal (Nemeroff, 1996). Stress is considered to be a major nongenomic factor that contributes to the expression or exacerbation of acute symptoms, recurrence or relapse after a period of remission, treatment outcome and failure to respond to pharmaco- and psychotherapy of psychiatric disorders (Maghaddam, 2002). The HPA-axis is the key system in controlling the stress response. Long-lasting hyper(re)activity of the CRF neurons, resulting in increased stress responsiveness and reflecting a glucocorticoid resistant state, is commonly seen in depressed individuals (Swaab et al., 2005). Observations in humans further indicate that aversive experiences, both in utero and in the neonatal period, result in sustained HPA-axis activation. Maternal stress beginning at infancy and subsequent stress during childhood is accompanied by a sensitization of the child's HPA-axis response to subsequent stress exposure. Stressful life events such as bereavement, child abuse, and early maternal separation are also risk factors for depression, anxiety disorder, or

both. Childhood physical or sexual abuse are important early stressors that may predispose individuals to adult onset depression accompanied by a permanent hyperactivity of the HPA system (Swaab et al., 2005).

On the other hand, the involvement of a GABAergic dysfunction in mood disorders, and subsequent impairment of central inhibition control has been suggested to be implicated in various psychiatric illnesses, syndromes and disorders affecting the CNS, particularly affective disorders. Thus, a reduced GABAergic activity in different cerebral regions accompanied by reduced GAD activity in depressive patients, more specifically decrease of $GAD_{65/67}$ in GABA neuronal density, has been observed (Sanacora et al., 1999). Clinical studies also reported low plasma and CSF GABA levels in mood disorder patients (Brambilla et al., 2003) and plasma GABA levels have been found to be lower in about 40% of depressed, manic, and euthymic subject (Hsu et al., 2003). $GABA_A$ receptor binding sites have been found to be abnormally increased in frontal cortex of depressed suicide victims, suggesting lowered GABAergic activity in those patients. In animal models of depression such as Forced swimming test, reduced GABA concentration in cerebral cortex and nucleus accumbens has been detected. Moreover, subsequent muscimol (a $GABA_A$ receptor agonist) administration in these animals increases the immobility time in Forced swimming test (Poncelet et al., 1987), whereas picrotoxin, a $GABA_A$ receptor antagonist, reduces the muscimol-induced reduction of immobility. Furthermore, $GABA_A$ receptors have also been found to be downregulated in the frontal cortex, hippocampus, and striatum of rats exposed to the learned helplessness paradigm (Brambilla et al., 2003), another experimental model of depression. Antidepressants, mood stabilizers, electroconvulsive therapy, and GABA receptor agonists have been shown to reverse the depression-like behaviour in animal models and to be effective in unipolar and bipolar patients by increasing brain GABAergic activity (Brambilla et al., 2003).

All these findings from available preclinical models are fairly consistent with a GABA transmission deficit or maladaptive response to stress, although classical tests such as forced swimming test or learned helplessness paradigm show certain disadvantages as short duration or extreme stress factors employed (e.g. electrical shock). Recent studies propose that models based on chronic stress aplication, such as maternal separation, reproduce depressive symptoms more concisely (Van Den Hove et al., 2005). It has been shown that prolonged periods (>1 h) of maternal separation (MS) during the first weeks of life result in animals with

behavioural and neuroendocrine signs of elevated stress reactivity as adults. In addition to an increase in immobility time in the Porsolt forced swimming test, anhedonia, and an enhanced anxiety-like behaviour, MS animals exhibit a dysfunction of the HPA axis reactivity to stress and therefore, the MS model in rat is considered nowadays as a robust model of enhanced stress responsiveness and depressive-like behaviour (Aisa et al., 2007).

Gender is one major variable that appears to confer differential vulnerability to stress. Men and women differ in physiological and behavioural responses to stressors and in epidemiologic patterns of stress-related illness. In humans, the prevalence of depression and anxiety disorders is clearly sex-dependent. Sex differences in sensitivity to stress also have been documented in animals. Male and female rats differ in numerous neuroendocrine and behavioural parameters, and vulnerability to stress is gender dependent. There is evidence for enhanced male susceptibility to posnatal enviromental manipulations. However, many of the relevant studies in the field of stress-related responses have been conducted in males, and less information is available on the response of female rodents to stressors (Aisa et al., 2007).

Taking into account all the above described, the objective of this project is to study effects of maternal separation on GABAergic system. Particular objectives of the project are:

1. To develop a model of maternal separation in rat. Depressive-like behaviour will be tested in the Porsolt forced swimming test.
2. To study the effect of maternal separation on GABAergic system. Levels of GABA and expression of the $GABA_A$ receptor will be determined in different cerebral regions.
3. To study gender-dependent differences in the behavioural and neurochemical processes induced by maternal separation.

III. MATERIAL AND METHODS

1. MATERIAL

1.1. Animals

All the experiments were carried out in strict compliance with the recommendations of the EU (DOCE L 358/1 18/2/1986) for the care and use of laboratory animals. Timed-pregnant Wistar rats were provided on gestation day 16 from Charles River Laboratories (Portage, MI, USA), individually housed in a temperature (21±1 °C) with air interchange (16 t/hour) and humidity (55±5 %) controlled room on a 12-h light/dark cycle with food and water freely available, in the animal-house establishment of the University of Navarra (C.I.F.A.). The experiments were performed between 9:00 a.m. and 14:00 p.m. to avoid the circadian rhythm influence. Pups were housed together with dams as described above except when stated.

1.2. Products and reagents

For the measurement of GABA levels by HPLC, the following products were used:

- pre-column derivatization: *o*- phthalaldehyde (OPA) and β- mercaptoethanol (BME), both supplied by Sigma-Aldrich Ltd (Germany).

- sample preparation: sodium tetraborate, supplied by Sigma-Aldrich Ltd.

Protein concentration was determined by Bradford technique using a "Kit for Protein Assay" (Bio-rad, Hercules, CA, USA).

In Western blot analysis the following products were used:

- NuPAGE® 10% Bis-Tris Gel 1.00 mm x 10 well; 20X NuPAGE® MES; 1X NuPAGE® SDS Running Buffer; NuPAGE® Antioxidant; NuPAGE® LDS Sample Buffer (4X); NuPAGE® Reducing Agent (10X) (Invitrogen, Carlsbad, CA, USA) .

- Immun-Blot® PVDF Membrane for Protein Blotting (Bio-Rad, Hercules, CA, USA).

- rabbit anti-$GABA_A$ receptor α_1 subunit polyclonal antibody (Ab5592, Chemicon International, EEUU).

- horseradish-conjugated polyclonal goat anti-rabbit immunoglobulins/HRT (DakoCytomation, Dinamarca).

- Anti-β-Tubulin III Antibody produced in rabbit (Sigma-Aldrich, Inc., Germany).

- Re-blot Plus Strong Solution 10X (Chemicon International).

- kit ECL™ (Amersham Bioscience, England) for membrane chemiluminescent revelation.

- Hyperfilm™ ECL films (Amersham Bioscience, England) for autoradiographic exposition.

All other chemicals and inorganic salts were supplied by Merck (Germany).

2. METHODS

2.1. Maternal separation

All litters were born within a 2-day period. As previously described (Huot et al., 2001; Ladd et al., 2000), on postnatal day (PND) 2, all pups were sexed and randomly assigned to the control group (NMS), pups were only briefly manipulated to change the bedding in their cages once weekly, or the separation group (MS), pups separated from their dam for 180 min from PND 2 to 21 inclusive. Before manipulation of the MS pups, each dam was removed from her home cage and placed in an adjacent cage. Pups were removed as complete litters, placed in an empty cage with standard bedding material and transferred to an incubator in an adjacent room. To compensate for the mother's body heat, the temperature of the incubator was adjusted to the age of the neonates: 32 ± .5°C (PND 2-5), 30 ± 0.5 ºC (PND 6-14) or 28 ± 0.5ºC (PND 15-21). Protocols involving manipulation of the pups took place between 10:00

and 13:00 h daily from PND2 to 21 inclusive. At the end of the separation period, foster litters were returned to their maternity cages, followed by reunion with the dams. Rats were weaned on PND 23 and were group-housed in same-sex, same-treatment-group until adulthood. All subsequent experiments were performed in adulthood (>60 days). Body weight was monitored at weekly intervals until adulthood.

2.2. Experimental design

Different subsets of rats were used for the measurement of each of the behavioural or biochemical parameter studied. Animals from different litters were distributed over the experiments. Adult NMS and MS rats were tested on the Porsolt forced swimming test. Two weeks later, animals were sacrificed and their brains collected.

2.3. Behavioural test

Porsolt forced swimming test (FST)

For the behavioural test, observer was blind to the rearing condition. The FST is a measure of behavioural despair that possesses high predictive validity and good face validity as a screen for depressive behaviour (Willner, 1984). As described by Porsolt et al. (1977), two swimming sessions were conducted: an initial 15-min pretest followed 24 h later by a 5-min test.

Rats were placed individually in a vertical Plexiglas cylinder (height: 45 cm, diameter: 19 cm) filled with 28–30 cm, a depth that rats could not touch the bottom with their hind paws, of 26 °C water. Immobility, regarded as the animal sign of depression (induced by effects of maternal separation experience), was considered as rats floating passively, making only small movements to keep its nose above the surface, as represented in Figure 7. Swimming was defined as time spent engaged in active swimming or struggling movement.

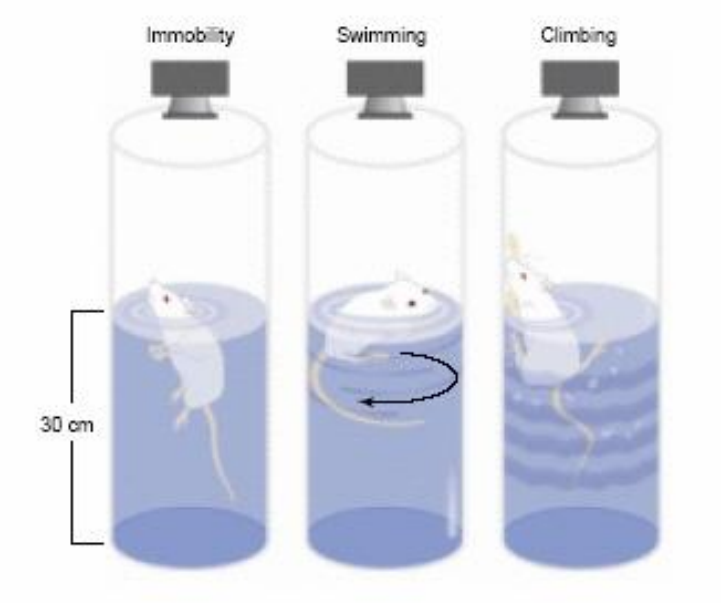

Figure 7. Representative cartoon of rats subjected to the Porsolt forced swimming test

2.4. Tissue collection

Rats were sacrificed by decapitation. Brains were removed and dissected on ice to obtain the frontal cortex, temporal frontex, striatum and hippocampus and frozen immediately on dry ice and stored at −80 °C.

2.5. GABA determination

GABA content was measured using high performance liquid chromatography (HPLC) with electrochemical detection (Waters Spheribor® 5µ ODS2 4.6x150 mm).

Samples of frontal and temporal cortex, striatum and hippocampus were homogenized with sonicator in 200 volumes of 0,1M sodium tetraborate at pH 9,1 and centrifuged at 11 000 rpm, 4°C, during 20 minutes (MPW Med. Instruments, Poland). GABA concentration in the supernatant was measured by HPLC pre-column including derivatization with *o*-phthalaldehyde (OPA) and β- mercaptoethanol (BME) (Roettger and Goldfinger, 1991). The mobil phase was composed of a mixture of phosphate buffer (0.1M NaH_2PO_4, pH 5.5) and methanol in ratio of 72:28 (v/v). GABA content was calculated by comparing with a 2 ng standard. The limit of detection was 50 pg/10 µl.

Chromatographic conditions

1) Isocratic mobil phase (720 ml of 0.1M NaH_2PO_4, pH 5.5; 280 ml of Methanol): this mixture was filtered and degassed through a 0.22 μm nitrocellulose membrane (Millipore, United Kingdom).

2) *Column:* Waters Spheribor® 5μ ODS2 4.6x150 mm

3) *Chromatographic equipment:*

- High pressure pump (Waters 515) of double pump piston with flow rate 1.4 ml/min
- Amperometric detector (DECADE, Antec-Leyden)
  Work electrode potencial: 0,7 V
  Sensitivity: 50 nA
  Column temperature (Toven): 40°C
- Injector (Waters 717plus Autosampler)
  Injection volume: 10 μl
  Dilution: 1:200 in TTB
- Stationary phase (AMINAS system, Millenium)

4) *pre-column derivatization:* The column was derivatized by *o*- phthalaldehyde (OPA) and β- mercaptoethanol (BME) in order to detect aminoacids in amperometric detector. The preparation of OPA-BME solution as follows: 9 ml of 0.1 M sodium tetraborate buffer, pH 9.1; 1 ml of OPA (27 mg/ml of ethanol); 11 μl of BME solution (10 μl of BME and 90 μl of sodium tetraborate buffer). OPA-BME solution was daily prepared and kept at room temperature. The injector was preset to mix the OPA-BME solution with the sample or standard solution in the same ratio (1:1). The sample was left to react with OPA during 1.5 min and then 20 μl of mixture was injected.

5) Standards: To prepare a 2 ng standard of GABA, 2 mg of GABA were dissolved in 1ml of deionized water, and afterwards, it was diluted twice consecutive in the ratio of 1:100. GABA was mixed. 20 μl of this mixture was injected. In Figure 8, a chromatogram shows the peaks obtained after injecting 20 μl of the standard solution.

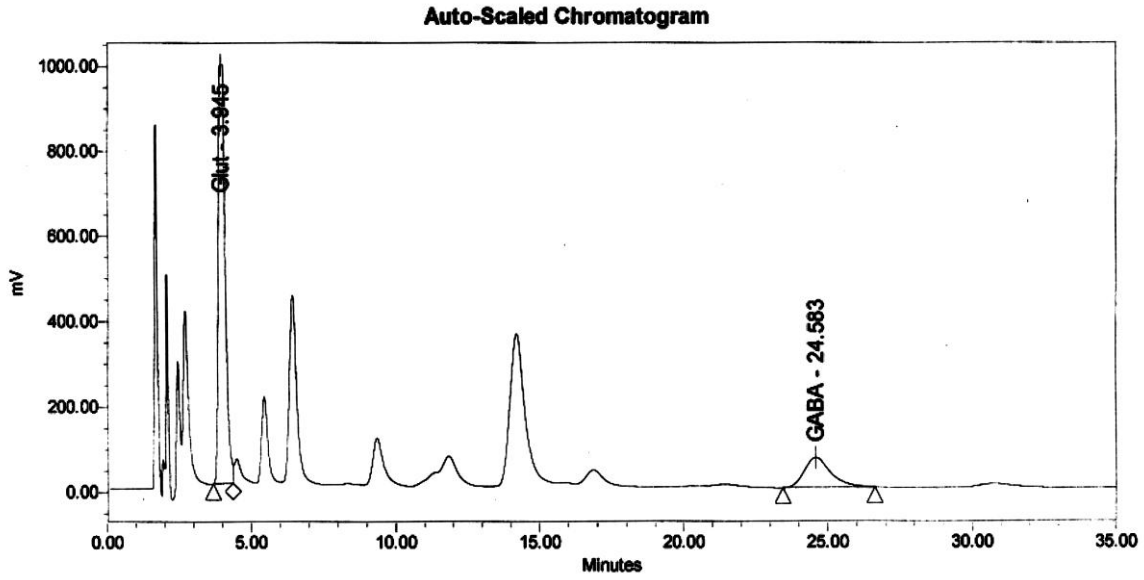

Figure 8. Representative chromatogram to detect GABA adjusted for identification and quantification of chromatographic peaks.

2.6. $GABA_A$ receptor expression: Western blot analysis

Western Blotting or immunoblotting allows to determine a certain protein and its relative amount in different samples. Hence, by taking advantage of distinct physical characteristics of different polypeptide species such as size, electrical charge, or shape, a complex mixture of proteins can be resolved electrophoretically by applying the sample to polyacryalmide gel matrix under the action of electric current and in the presence of sodium dodecyl sulfate (SDS). SDS resolves the proteins roughly according to their relative molecular weight (and to some degree, shape) regardless of charge. The samples are prepared from tissues that are homogenized in gel loading buffer that protects the protein of interest from degradation. Then transfer of the proteins fractionated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) to a solid support membrane (Western blotting) follows. Once the proteins have been transferred to the solid support membrane, the membrane is referred to as a "blot". Afterwards, the blot is incubated with a generic protein (such as milk proteins) to bind to any remaining sticky places on the membrane. A primary antibody is then added to the solution which is able to bind to its specific protein. A secondary antibody which recognizes the primary antibody is added to find locations where the primary antibody bound. The secondary antibody is labelled by conjugating them with an enzyme such as alkaline phosphatase or horseradish peroxidase, which can be detected visually through the conversion

of a colorimetric substrate (chromagen) to a colored precipitate at the site of antibody binding. Radioactive isotope labelling can be also used as detection methods, as well as other, non-radioactive techniques.

2.6.1. Preparation of the membrane protein extracts

The quantification of $GABA_A$ receptor levels were realized in membrane extracts from frontal and temporal cortex, striatum and hippocampus. Tissue was resuspended in 10 volumes of the ice-cold homogenization buffer (TRIS-HCl 50mM; pH=7.4; EDTA 500mM; PMSF 1mM; aprotinine 2.1 mg/ml; leupeptine 1mg/ml; sucrose 1.1g), homogenized with sonicator, and centrifuged at 2,000 rpm, 4°C, for 10 minutes. The supernatant was collected and centrifuged at 10,000 rpm, 4°C, during 20 minutes once again. Afterwards, pellets were homogenized in lysis buffer and once more centrifuged at 10,000 rpm, 4°C, during 20 minutes. In the end, the target pellets were used for future manipulation and resuspended in 50 µl of homogenization buffer of composition above described. The final concentration of the protein in a 5 µl sample was determined spectrophotometrically using Bradford technique.

2.6.2. Western blotting

Samples containing 10 µg of protein from frontal cortex, temporal cortex, striatum and hippocampus were loaded onto 10% NuPAGE® Pre-cast Gels (NuPAGE® 10% Bis-Tris Gel 1,00 mm x 10 well) and separated by SDS-PAGE during 40 minutes at 200 V in SDS running buffer (prepared by adding 25 ml 20X NuPAGE® MES to 475 ml of deionized water, with the lower buffer chamber filled with 300 ml 1X NuPAGE® SDS Running Buffer and the upper buffer chamber filled with NuPAGE® SDS Running Buffer containing 500 µl NuPAGE® antioxidant). The total volume of sample was 10 µl, composed of NuPAGE® LDS Sample Buffer, NuPAGE® Reducing Agent and distilled water. Samples were run under reducing and denaturing conditions (NuPAGE® LDS Sample Buffer, NuPAGE® Reducing Agent) and were heated at 70°C for 10 minutes prior to loading gel. Separated proteins, previously soaked in transfer buffer (500 ml of distilled water; 3.00 g of amoniacal TRIS and 1.54 g of boric acid) were electrophoretically transferred from gels to PVDF membranes during 30 minutes at 25 V. Then the membrane was incubated with blocking protein solution (10% non-fat dried milk, phosphate buffered saline (PBS) and 0.t1% Tween 20) for one hour at room temperature with agitation. After decanting the blocking buffer from the blot, the membrane was incubated with

diluted primary antibody (1:5,000 equally for all the cerebral regions) overnight at 4°C with gentle agitation.

$GABA_A$ receptor protein was detected with a rabbit anti-$GABA_A$ receptor α_1 subunit polyclonal antibody. After incubation with the primary antibody for 24 hours, the blot was washed three times with phosphate buffer saline containing 0.1% Tween (PBST) before addition of secondary antibody (polyclonal goat anti-rabbit immunoglobulins/HRT) 1: 10,000, diluted in 10% blocking protein solution and incubated for two hours at room temperature with agitation. Before the blot was revealed, it was washed three times in PBST and once in PBS. Immunopositive bands were visualized by a chemiluminescent method (kit^{ECL}).

One lane of the blot is reserved for a "marker," or ladder,"a commercially available mixture of proteins having defined molecular weights. Size approximations are taken by comparing the stained bands to that of the marker or the ladder loaded during electrophoresis. The process is repeated for a structural protein, such as tubulin that should not change between samples. The amount of target protein is indexed to the structural protein on the membrane in case of errors in sample loading or incomplete transfers. In this case, the membrane was stripped with a stripping buffer (Re-blot Plus Strong Solution 10X) at room temperature for 30 min, and re-labeled with the primary antibody against tubulin (1:25,000 dilution).

The optical density of $GABA_A$ receptor-reactive bands (~51 kDa) (Figure 9) visible on x-ray film ($Hyperfilm^{TM}$ ECL) was determined densitometrically (Murphy et al., 2002) using ImageMaster I-D program (Pharmacia).

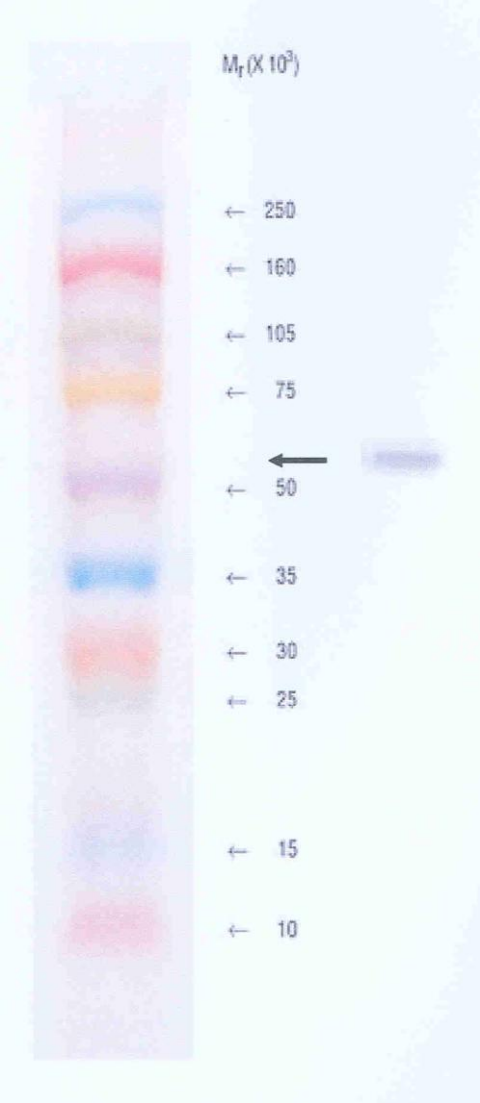

Figure 9. Picture showing a representative autoradiograph hybridization signal obtained by the western blot technique using a specific anti-$GABA_A$ receptor α_1 subunit antibody. Bands corresponding to $GABA_A$ receptors were observed at a molecular weight of 51 kDa (close to the violet colour of the molecular marker).

2.7. Data analysis

Data were analysed by SPSS for Windows, release 11.0. Normality was checked by Shapiro-Wilks´s test ($p>0.05$). Behavioural and biochemical data were analysed by unpaired *t*-Student tests (to compare NMS vs MS groups). Data are presented as mean ± SEM and the level of significance was set at $p< 0.05$.

IV. RESULTS

1. BEHAVIOURAL CHARACTERIZATION OF MATERNAL SEPARATION

1. 1. Endophenotype

At the end of the maternal separation period (PND 21), body weight of the MS pups was significantly lower (Student´s t-test, $p<0.05$, n=) than control pups (Figure 10) both in males and females.

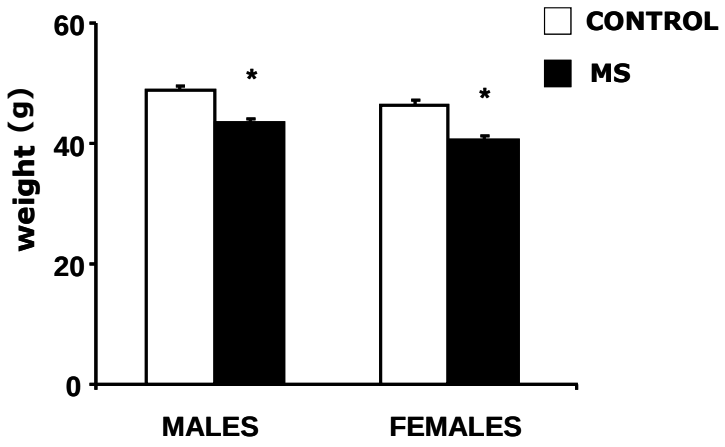

Figure 10. Body weight of pups at the end of the maternal separation (MS) period. *p< 0.05 (Student's *t*-test).

However, when adults (PND 75), there were no differences in body weight between MS and control rats. As expected, males exhibited overall higher body weight than females (Figure 11).

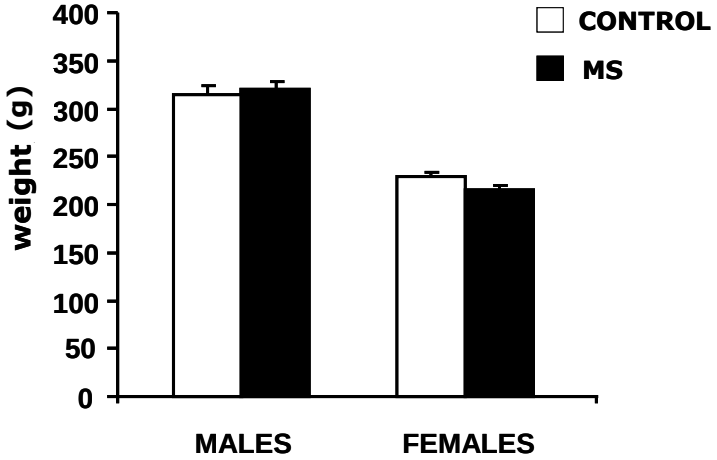

Figure 11. Body weight of adult control and maternal separation (MS) rats.

At gross examination general physical condition of the MS offspring was not affected. Adult MS rats showed no signs of irritability, agressive behaviours, or other behavioural changes. Exploration and locomotion seem also similar to controls.

1.2. Porsolt forced swimming test

Immobility times in the Porsolt forced swimming test as representative of depressive-like behaviour are shown in figures 12 and 13.

In males, MS (n=10) produced a significant increase in immobility compared with control (n=10) rats (Student's *t*-test, $p<0.05$).

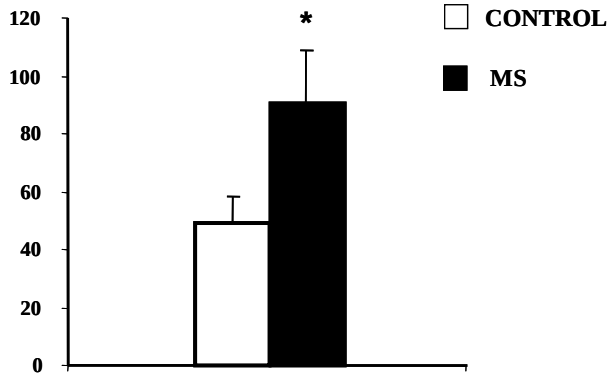

Figure 12. Behavioural characterization of maternal separation (MS) rats: effects of MS in the Porsolt forced swimming test in male rats. Data are presented as mean ± SEM; and the level of significance was set at $*p< 0.05$ (Student's *t*-test).

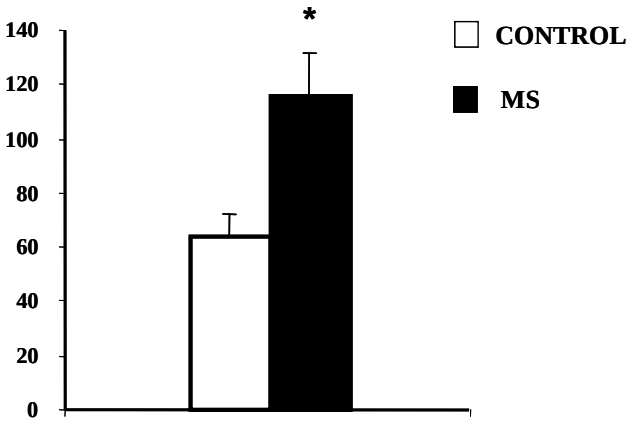

Figure 13. Behavioural characterization of maternal separation (MS) rats: effects of MS in the Porsolt forced swimming test in female rats. Data are presented as mean ± SEM; and the level of significance was set at $*p< 0.05$ (Student's *t*-test).

Similarly, female rats exhibited a significant increase in immobility time (Student's *t*-test, $p<0.05$, n=10 each group). Compared to males, immobility time were higher in female rats.

2. EFFECT OF MATERNAL SEPARATION ON THE GABAERGIC SYSTEM

2.1. GABA levels

Male rats

GABA content, measured in homogenates from male frontal cortex, was significantly higher in MS rats compared to controls in the frontal cortex (Student's *t*-test, $p<0.05$, Figure 14). However, no differences between the MS and control group were found in the temporal cortex (Figure 15).

As shown in Figure 16, a significant decrease (Student's *t*-test; $p < 0.05$) in endogenous GABA levels in rats subjected to MS compared to control animals was found in the striatum.

No significant alterations in total GABA content in hippocampus was found in the MS group when compared to controls (Figure 17).

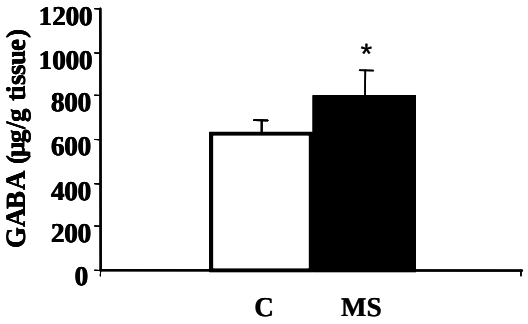

Figure 14. GABA content in homogenates from male rat frontal cortex. C, control; MS, maternal separation. Data are expressed as mean ± SEM (n = 8 – 12). The level of significance was set at *$p< 0.05$ in Student's *t*-test.

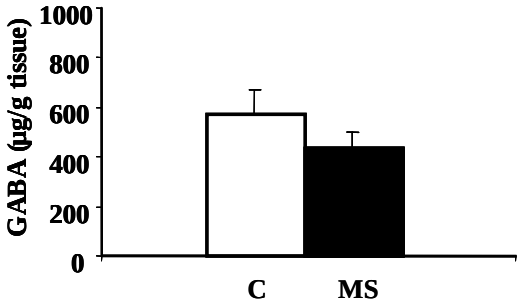

Figure 15. GABA content in homogenates from male rat temporal cortex. C, control; MS, maternal separation. Data are expressed as mean ± SEM (n = 8 – 12).

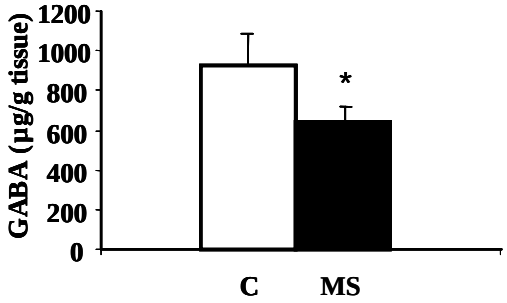

Figure 16. GABA content in homogenates from male rat striatum C, control; MS, maternal separation. Data are expressed as mean ± SEM (n = 9 – 10). The level of significance was set at *$p < 0.05$ in Student's *t*-test.

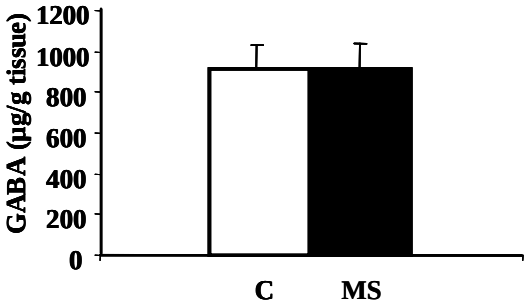

Figure 17. GABA content in homogenates from male rat hippocampus. C, control; MS, maternal separation. Data are expressed as mean ± SEM (n = 9 – 12).

Female rats

Figures 18, 19, 20 and 21 show total GABA content in homogenates from the frontal cortex and temporal cortex, striatum and hippocapus of female rats.

In the frontal cortex, and at similarity to males, a significant increase in GABA levels (Figure 18; Student's *t*-test; $p < 0.05$) has been observed in animals subjected to MS compared to control animals

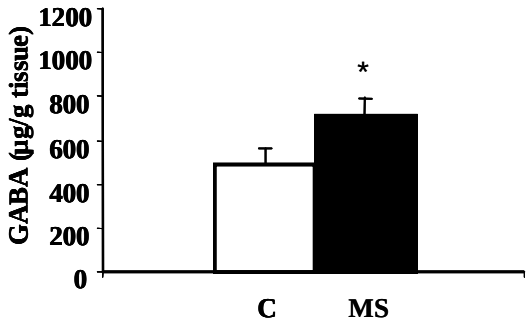

Figure 18. GABA content in homogenates from female rat frontal cortex**.** C, control; MS, maternal separation. Data are expressed as mean ± SEM (n = 8 – 10). The level of significance was set at **p*< 0.05 in Student's *t*-test.

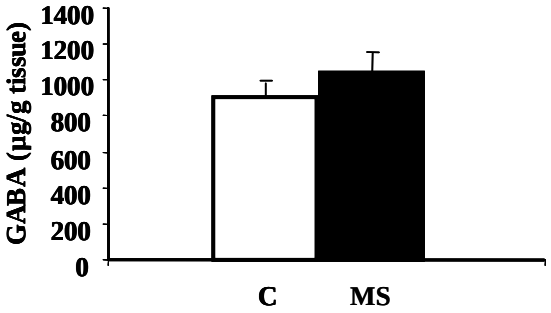

Figure 19. GABA content in homogenates from female rat temporal cortex**.** C, control; MS, adult male rats subjected to maternal separation. Data are expressed as mean ± SEM (n = 8 – 10).

No other significant alterations in GABA levels have been found between control and MS groups in any of the other regions studied (Figures 19, 20 and 21).

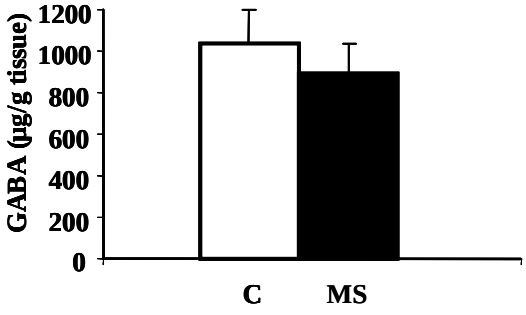

Figure 20. GABA content in homogenates from female rat striatum C, control; MS, maternal separation. Data are expressed as mean ± SEM (n = 7 – 12).

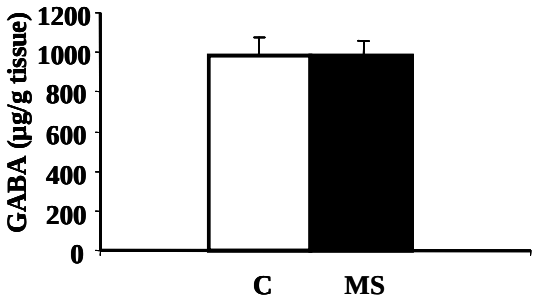

Figure 21. GABA content in homogenates from female rat hippocampus C, control; MS, maternal separation. Data are expressed as mean ± SEM (n = 8).

2.2. $GABA_A$ receptor quantification

β-tubulin III was used as an internal control of protein load. As shown in the next Figure 22, protein load was similar in all lanes.

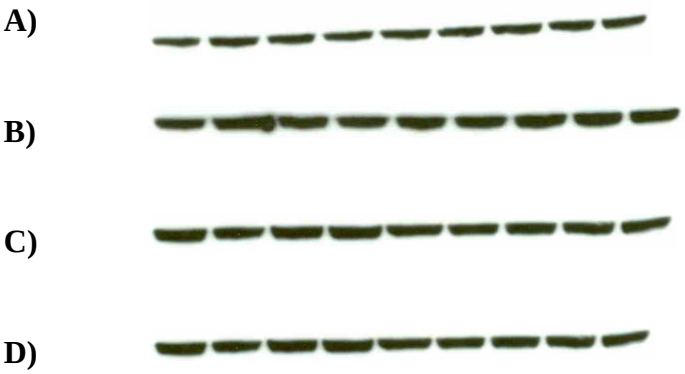

Figure 22. β-tubulin III, an internal control of protein load in frontal cortex (A), temporal cortex (B), striatum (C) and hippocampus (D). Apparent molecular mass < 60 kDa.

Male rats

A significant decrease in $GABA_A$ receptor expression (α_1 subunit) in the frontal cortex (Student's *t*-test; p = 0.001) was observed in animals subjected to maternal separation (Figure 23). In the temporal cortex, no differences were found in the expression of $GABA_A$ receptor when comparing MS (n = 6) rats to controls (n = 6) (Figure 24).

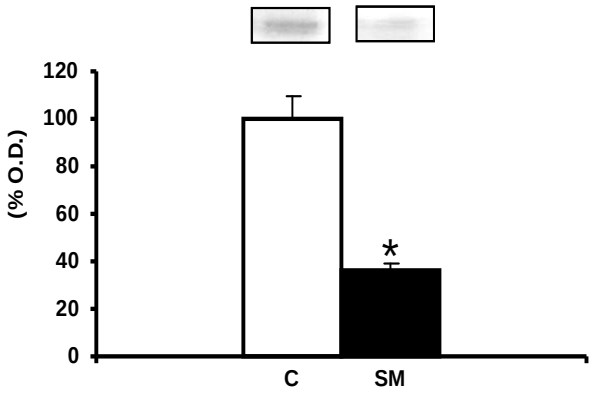

Figure 23. $GABA_A$ receptor expression in the frontal cortex of male rats. C, control; MS, maternal separation. Data are expressed as percentage of optical density (O.D.) values of control rats (mean ± SEM). The level of significance was set at *p< 0.05, Student's *t*-test.

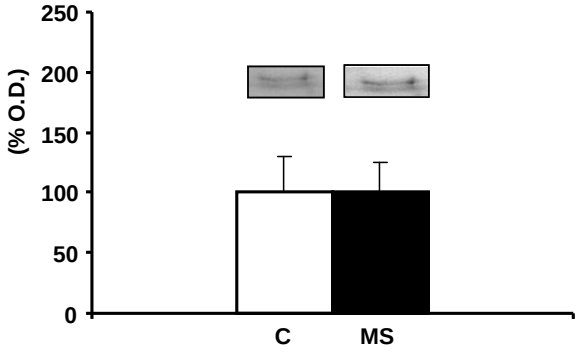

Figure 24. $GABA_A$ receptor expression in the temporal cortex of male rats. C, control; MS, maternal separation. Data are expressed as percentage of optical density (O.D.) values of control rats (mean ± SEM).

In the striatum, as shown in Figure 25, a significant increase in $GABA_A$ receptor expression in the MS group (n = 6) compared to control (n = 6) was found (Student's *t*-test; $p < 0.015$).

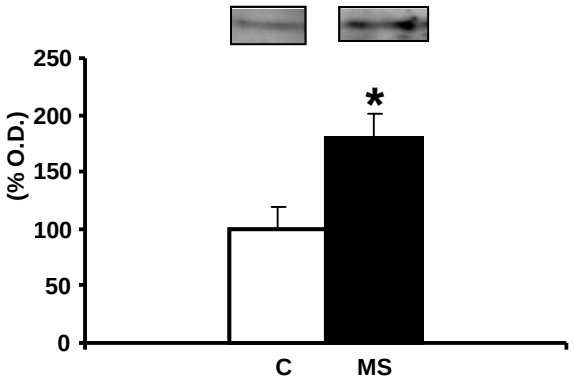

Figure 25. $GABA_A$ receptor expression in the striatum of male rats. C, control; MS, maternal separation. Data are expressed as percentage of optical density (O.D.) values of control rats (mean ± SEM). The level of significance was set at *p< 0.05, Student's *t*-test.

In the hippocampus (Figure 26), no differences in expression of $GABA_A$ receptor were measured comparing controls (n = 6) to the MS (n = 6) groups .

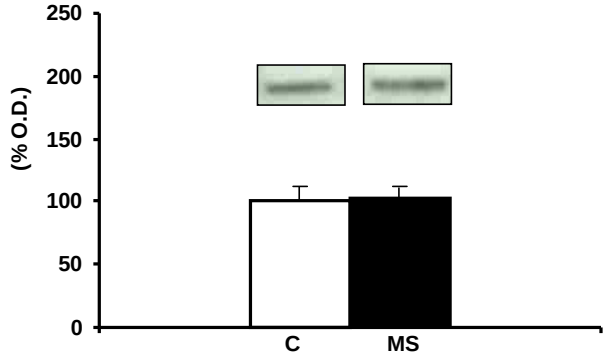

Figure 26. $GABA_A$ receptor expression in the hippocampus of male rats. C, control; MS, maternal separation. Data are expressed as percentage of optical density (O.D.) values of control rats (mean ± SEM).

Female rats

In frontal cortex of female rats (Figure 27), a significant decrease in $GABA_A$ receptor expression in MS group (n = 6) compared to controls (n = 6) was found (Student's *t*-test, p = 0.037), whereas no differences were observed in the temporal cortex (Figure 28). These reults are similar to those found in male rats.

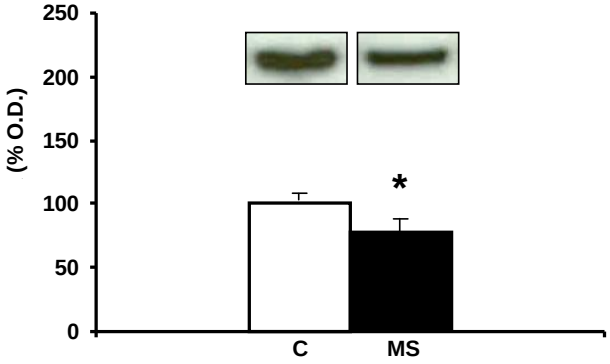

Figure 27. $GABA_A$ receptor expression in the frontal cortex of female rats. C, control; MS, maternal separation. Data are expressed as percentage of optical density (O.D.) values of control rats (mean ± SEM). The level of significance was set at $*p < 0.05$, Student's *t*-test.

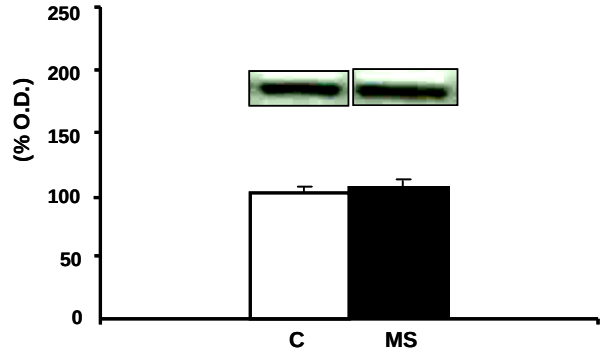

Figure 28. $GABA_A$ receptor expression in the temporal cortex of female rats. C, control; MS, maternal separation. Data are expressed as percentage of optical density (O.D.) values of control rats (mean ± SEM).

No differences in $GABA_A$ receptor expression between C (n = 6) and MS groups (n = 6) were observed in the striatum (Figure 29).

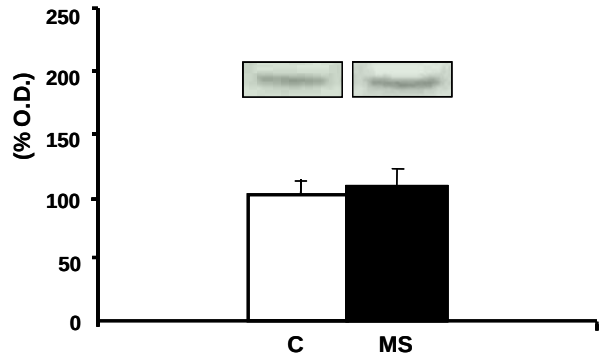

Figure 29. $GABA_A$ receptor expression in the striatum of female rats. C, control; MS, maternal separation. Data are expressed as percentage of optical density (O.D.) values of control rats (mean ± SEM).

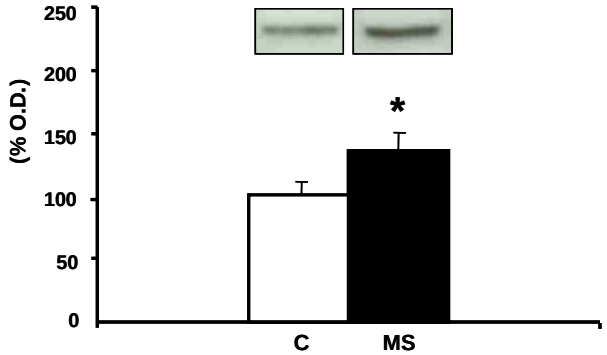

Figure 30. $GABA_A$ receptor expression in the hippocampus of female rats. C, control; MS, maternal separation. Data are expressed as percentage of optical density (O.D.) values of control rats (mean ± SEM). The level of significance was set at $*p < 0.05$, Student's *t*-test.

As shown in Figure 30, in the hippocampus, a significant increase of $GABA_A$ receptor expression in MS group (n = 6) compared to control (n = 6) was measured (Student's *t*-test, p = 0.049).

V. DISCUSSION

Depression is a common mental disorder, and currently a major public health concern. Although the underlying neurobiology of depression remains elusive so far, there is an increasing evidence implicating stress in brain disturbances thought to underlie certain forms of depression or particular components of the depressive syndrome (Van Praag, 2004; Kendler et al., 1999; Kessler, 1997).

The hypothalamic–pituitary–adrenal (HPA) axis is an essential component of an individual's capacity to cope with stress and in fact, a hyperactivity of the HPA axis is observed in the majority of patients with depression (de Kloet et al., 2005; reviewed by Arborelius et al., 1999). Stress stimulation of the axis starts when corticotropin releasing factor (CRF) released by the paraventricular nucleus of the hypothalamus (PVN) stimulates the release of corticotropin (ACTH) from the anterior pituitary, which in turn, stimulates the secretion of glucocorticoids from the adrenal cortex. Many of the behavioural consequences of stress are thought to be mediated by the activation of the glucocorticoid receptors by stress-induced high levels of glucocorticoid hormones (Roozendaal et al., 2006a; Oitzl et al., 2001; de Kloet et al., 1998) and subsequent alteration in gene expression (see review by Berton and Nestler, 2006). There are also modulatory influences, mainly glucocorticoid negative feedback that inhibits CRF synthesis and release, thus dampening HPA responses to stress (De Kloet et al., 1998). The hippocampus, which exhibits a high density of corticosteroid receptors, plays an important role in the negative regulation of the HPA axis (Jacobson and Sapolsky, 1991).

Inevitably, depression investigations have been limited by the lack of good animal models. There is compelling evidence that exposure to early stressful adverse life events may increase vulnerability to psychopathology in adult life. This association has led to the belief that stress or early adverse experiences program changes in the brain, which persist throughout lifetime and predispose an individual to the development of depression (Heim and Nemeroff, 2001). In fact, individuals who experience early trauma, such as parental loss, sexual abuse or physical assault in childhood, present an increased risk for suffering from depression later in life (Heim and Nemeroff, 2001). McCauley et al (1996) reported that women who experienced child abuse exhibited a four-fold higher risk to suffer from depression in adulthood compared to naive women. Moreover, a study performed by Ladd et al (2000) indicated that women that suffered from sexual or physical child abuse experience exhibited a permanently hypersensitive system of response to stress.

Based on these arguments, rat models of early life adversity have been developed, including those in which the neonatal animals are periodically deprived of contact with the dam, usually known as maternal separation (MS). MS alters the HPA function and the ability of the organism to respond to, cope with and adapt to stressful stimuli. It has been shown that prolonged periods (>1 h) of MS during the first weeks of life result in behavioural and neuroendocrine signs of elevated stress reactivity in animals as adults (Ploj et al., 2003; Ladd et al., 2000; Lehman and Feldon, 2000; Anisman et al., 1998). In addition to an increase in immobility time in the Porsolt forced swimming test, anhedonia, and an enhanced anxiety-like behaviour, MS animals exhibit a dysfunction of the HPA axis reactivity to stress and therefore, the MS model in rat is considered nowadays as a robust model of enhanced stress responsiveness and depressive-like behaviour (Van Den Hove et al., 2005; Ladd et al., 2000). In contrast, rat pups separated for a short period (3-20 min, also known as neonatal handling) from their dam during the neonatal period, exhibit the opposite effects (Ploj et al., 2003).

The length of daily maternal separation prior to weaning is important in determining the nature of neuroendocrine and behavioural effects. In the rat, the postnatal development of the stress system is characterized by a so-called stress hyporesponsiveness period (Schapiro et al., 1962), lasting from about postnatal day 4 to 14. Rat pups during the stress hyporesponsiveness period exhibit low basal concentration of corticosterone and an inability of mild stressor to induce a corticosterone response (Levine, 1994). We developed a maternal separation model of depression, in which pups were periodically deprived of contact with the dam for 180 min a day from PND 2 to 21. In a previous work from the laboratory (Aisa et al., 2007), it was described that, compared to normally reared animals, MS rats show in adulthood depressive-like behaviour in the forced swimming test (Ladd et al., 2000; Hall, 1998; Plotsky et al., 1998; Willner., 1990), anhedonic behaviour (Huot et al., 2001; Zurita and Molina, 1999; Willner et al., 1987) and anxiety behaviour (Huot et al., 2000; Wigger and Neumann, 1999), increased HPA axis responsiveness to stressors (Wigger and Neumann, 1999; Ladd et al., 1996; Plotsky and Meany, 1993; Rosenfeld et al., 1992) and elevated CRF mRNA in the PVN (Ladd et al., 1996; Plotsky and Meany, 1993). Therefore, neonatal MS in the rat can be considered as an animal model of vulnerability to development of depression-like syndrome and an enhanced stress responsiveness (Ladd et al., 2005; Sanchez et al., 2001). It can be suggested that alterations in the behavioural phenotype associated to stress are related to the increased HPA axis responsiveness to stressors, as the depressive-like behaviour was reversed by administering the glucocorticoid receptor antagonist mifepristone (Aisa et al., 2007). In

this regard, in clinical studies, it has been recently shown that by regulating the HPA axis, mifepristone may be effective in the treatment of psychotic major depression (Flores et al., 2006) and bipolar disorder (Young et al., 2004).

Following the above described MS protocol (Aisa et al., 2007), and to confirm the depressive-like behaviour, immobility time in the Porsolt swim test (FST) was measured. The increase in immobility time in the FST is considered to reflect depressive behaviour as the animals seem to dispair to escape from the plexiglas cylinder. FST is suggested to be one of the most specific tests for detection of antidepressant effects (Lucki, 1997), producing these drugs a decrease of the immobility time. It was found that immobility time was significantly higher in the MS group, both in male and female rats. Therefore, it can be concluded that the MS model used in the present study could represent a valid model of depressive-like behaviour, both in male and female rats.

Following the monoaminergic hypothesis of depression, alterations in both the serotonergic and/or noradrenergic systems have been associated to the neurochemical mechanisms underliing depression. Nowadays, however, as described in the Introduction of the present project, other neurotransmitter systems have been suggested to be implicated in the etiology of depression. 27 years ago, the GABAergic hypothesis of mood disorders was formulated by Emrich et al. (1980). After Emrich's hypothesis, several studies have evaluated the potential role of GABAergic abnormalities in the pathophysiology of mood disorders and alterations in GABAergic transmission have been observed in mood disorder patients. Petty (1995) suggested that a hypofunction in the GABAergic transmission may lead to vulnerability of an individual to suffer from affective disorders. As revised by this author, certain enviromental factors can increase the extracelular levels of GABA, resulting in depressive symptoms. Furthermore, it has been observed that the GABA levels are increased within the luteal phase of menstruation period that could explain the increased prevalence of depression in women compared to men.

In the present work, differential changes in GABA levels according to gender and brain region studied have been found. It has been previously described that different brain regions are associated to stress response. In studies employing magnetic resonance, it has been suggested that alterations within the striatum, amygdala, and prefrontal cortex regions appear early in the course of bipolar disorder, so they seem to appear prior to the disease onset.

Similarly, studies using various methods have documented limbic and fronto-striatal dysfunctions in elderly depressive patients, as well as in young depressive patients with no other neurological pathology. In addition, it has been determined that the above cited dysfunctions could be associated to the short or long-term old-age depression (Alexopoulos, 2002). It has also been observed that infralimbic and prelimbic regions of the rat ventral medial prefrontal cortex seem to be involved in the detection of stressors which is considered to be a control mechanism to inhibit the stress-induced neuronal activity in brain stem nucleus (Amat et al, 2005).

Another important cerebral region is the hippocampus. In the hippocampal region, stress induced changes which may be involved in mood disorders have been observed. The hippocampus that exhibits a high density of corticosteroid receptors, plays an important role in the negative regulation of the HPA axis (Jacobson and Sapolsky, 1991). Plasticity of hippocampal circuitry may increase its vulnerability to various insults including stress. The majority of hippocampal granule neurons develops and extends their axons between postnatal days 1 and 21 (Amaral and Dent, 1981). This peak period of neurogenesis overlaps the stress hyporesponsiveness period (postnatal days 4-14). In this sense, MS rats have been described to exhibit decreased mossy fibre density in the stratum oriens region of the hippocampus (Huot et al., 2002). Therefore, MS during critical periods of hippocampal development can disrupt hippocampal cytoarchitecture in a stable manner. Recent studies performed by Chun-Hsu et al (2003) demonstrate an extreme sensibility of hippocampal GABAergic system to manipulations within the neonatal period.

It has been found both in male and female MS rats decreases in GABA levels in the frontal cortex accompanied by a decreased expression of the $GABA_A$ receptor, as already shown in a previous work, in which rats subjected to MS exhibited lower levels of both $GABA_A$ receptor and BDZs binding sites in various cerebral regions such as frontal cortex (Caldji et al., 2000). For the present study on the effects of on $GABA_A$ receptor expression it has been chosen the α_1 subunit as the $GABA_A$ receptor quantification marker due to its abundant (almost 90%) expression in rodent brain compared with β and γ subunits expression (Benke et al, 2004).

It could be suggested that increases in the neurotransmitter levels in the frontal cortex is a compensatory mechanism to the decreased receptor expression. Similarly, it could be

hypothesized that the increased levels of $GABA_A$ receptor expression in the striatum in males, could compensate for the decreased GABA levels in this region.

Concerning gender, one of major variable that appears to confer differential vulnerability to stress (see Objectives), we have observed an important gender difference regarding alterations in the GABAergic system associated to MS in the hippocampus. In females, significant increases in $GABA_A$ receptor levels were measured in the MS group, although no changes in GABA levels were found associated to the MS procedure. Qin et al (2004) performed a study in which rats after chronic stress exposure (three weeks) and subsequent administration of a high dose of corticosterone, exhibited an increase in α_1 $GABA_A$ receptor subunit expression in hippocampus. The study of Qin could support our data, as it has been described that MS effects on behaviour are related to increased corticosterone levels. It could be hypothesised that MS female rats would be more vulnerable to stress when adults due to these higher expression of $GABA_A$ receptors, as Petty (2005) described that in situations of increased inhibitory GABAergic tone, result in depressive symptoms.

It could only be speculated on the mechanisms involved in the GABAergic implication on MS effects. Cerebral regions such as frontal cortex and hippocampus exert inhibitory effects on the HPA axis activity related to the glucocorticoid feedback mechanism through the activation of glucocorticoid and mineralocorticoid receptors. The above cited brain regions regulate the HPA axis function via the glutamatergic transmission in terminal stria, medial preoptic area and hypothalamic ventromedial nucleus, in which GABA is the main neurotransmitter system (Conn et al, 2000; Kollack-Walker et al, 2000). Kovack et al (2004) showed an essential role of the GABAergic system as a negative regulator of neuronal excitability in the paraventricular nucleus (PVN). PVN neurons express CRF and integrate excitatory and inhibitory signals leading to consequent alterations in ACTH secretion. Moreover, it has been observed that CRF inhibits serotonergic neurons in the raphe nucleus and these neurons may regulate the stress response through efferent projections towards the PVN, amygdala and hippocampus. In addition, increasing evidence seem to suggest that GABAergic neurons localized in these cerebral structures exert inhibitory actions in both central CRF neurons and stress sensibility (Kaufmann et al, 2000). Prefrontal cortex is another region involved in the regulation of emotional responses that seem to be involved in the control of stress response at both behavioural and neuroendocrine levels (pituitary-adrenal).

This control seem to be exerted through inhibitory projections to the amygdala and hypothalamus (Davidson et al, 2000).

In summary, MS has been used to model changes in behaviour associated with exposure to early-life stress and altered HPA responses, both in males and females. Significant changes in GABAergic system, both in terms of neurotransmiter level and $GABA_A$ receptor expression have been found. However, differential results according to gender and brain region studied have been observed. Therefore, it can be suggested the implication of the GABAergic system in the depressive-like behaviour acompanying altered responses to stress induced by MS. Altogether, the present results challenges for future investigation of the implication of the GABAergic system in the etiopathogenic mechanisms involved in development of depression. It could also be speculated that behavioural phenotypes related to an altered HPA axis reactivity, such as depressive syndromes, could also benefit of future therapies including drugs acting on $GABA_A$ receptors.

VI. CONCLUSIONS

1. Maternal separation (MS) has been used to model changes in behaviour associated with exposure to early-life stress and altered HPA responses. Both male and female MS rats display in adulthood depressive-like behaviour in the forced swimming test as shown by significant increases in immobility time.

2. It has been found both in male and female MS rats, increases in GABA levels in the frontal cortex accompanied by a decreased expression of the $GABA_A$ receptor. In the temporal cortex, no differences between the MS and control group were found neither in terms of gender differences nor in terms of GABAergic system markers (GABA levels and $GABA_A$ receptor expression)

3. Important gender difference regarding alterations in the GABAergic system associated to MS have been found in the striatum and hippocampus. In the striatum of MS male rats there were increased levels of $GABA_A$ receptor expression that could compensate for the decreased GABA levels in this region. In the hippocampus of females, significant increases in $GABA_A$ receptor levels were measured in the MS group, although no changes in GABA levels were found associated to the MS procedure.

4. In conclusion, MS has been used to model changes in behaviour associated with exposure to early-life stress both in male and females. Significant changes in GABAergic system, both in terms of neurotransmiter level and $GABA_A$ receptor expression have been found. However, differential results according to gender and brain region studied have been observed. It can be suggested the implication of the GABAergic system in the depressive-like behaviour acompanying altered responses to stress induced by MS.

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SUMMARY IN CZECH (SOUHRN PRÁCE)

Tato práce se zabývá zapojením GABAergního systému do změněné reakce na stres vyvolané maternální separací u potkana.

Cílem práce bylo studovat vliv maternální separace na GABAergní systém, přesneji na biochemické markery tohoto systému (hladiny GABA neurotransmiteru, exprese $GABA_A$ receptorů), a studovat na pohlaví závislé rozdíly v chování a neurochemických procesech u potkana indukované maternální separací.

Aby bylo možné sledovat výše zmíněné cíle, nejprve bylo nutné vyvinout model maternální separace u potkana. Mláďata potkana byla separována od matky po dobu tří hodin denně od postnatálního dne 2 do postnatálního dne 21 (v postnatálním období, kdy jsou snížené schopnosti reagovat na stresove stimuly). V postnatální den 23 byla odstavena, rozdělena do skupin stejného pohlaví a bez významných manipulací ponechána vyrůst do dospělosti. Po dosažení dospělosti (> 60 dní) byli potkani rozděleni do dvou skupin. První skupina byla usmrcena dekapitací a jednotlivé regiony mozkové tkáně (přední a spánková kůra mozková, corpus striatum a hippocampus) byly použity ke stanovení hladin GABA vysokoúčinnou kapalinovou chromatografií a ke stanovení míry exprese $GABA_A$ receptorů western blot technikou. Druhá skupina byla podrobena Porsolt forced swimming testu a 14 dní po experimentu usmrcena rovněž dekapitací.

Maternální separace byla použita k napodobení změn v chování spojených s expozicí stresu v časném období života a změněné HPA odpovědi. Jak maternálně separovaní samci tak samice potkana vykazují v dospělosti znaky depresivního chování v Porsolt forced swimming testu, což se odráží v signifikantním vzrůstu nehybnosti laboratorního zvířete při tomto experimentu. Při stanovení výše hladin GABA HPLC byly, jak u maternálně separovaných samců, tak samic potkana pozorovány zvýšené hladiny GABA v přední kůře mozkové doprovázené sníženou expresí $GABA_A$ receptoru. Ve spánkové kůře nebyly nalezeny žádné rozdíly mezi maternálně separovanými a kontrolními skupinami potkanů ani mezi jednotlivými pohlavími, ani ve smyslu markerů GABAergního systému. Významné rozdíly mezi pohlavími týkající se změn v GABAergním systému přičítané maternální separaci byly nalezeny v corpus striatum a hippocampu. V corpus striatum maternálně separovaných samců byly stanoveny zvýšené hladiny exprese $GABA_A$ receptoru které by mohly kompenzovat snížené hladiny GABA v tomto regionu. V hippocampu maternálně separovaných samic bylo naměřeno významné zvýšení exprese

$GABA_A$ receptoru, ačkoli žádné rozdíly v hladinách GABA ve spojení s maternální separací nebyly naměřeny.

Výsledky dosažené v tomto projektu tedy podporují domněnku, že GABAergní systém hraje roli ve vzniku depresivního syndromu, a že by se mohl podílet na změněné odpovědi na stresové podněty vyvolané procesem maternální separace. Dosavadní výsledky tak představují výzvu pro budoucí výzkum úlohy GABAergního systému v etiopatogenických mechanismech účastnících se vzniku deprese.