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Jordan MARROCCO

NOVEL INSIGHTS IN THE GLUTAMATERGIC HYPOTHESIS OF DEPRESSION: A NEUROCHEMICAL AND PHARMACOLOGICAL STUDY IN THE RAT MODEL OF PRENATAL RESTRAINT STRESS

Members of the Jury

Dr. Jean-Claude MICHALSKI	CNRS-University of Lille 1, France	***President of the Jury***
Prof. Anna PITTALUGA	University of Genoa, Italy	***Examiner***
Dr. Harm J KRUGERS	University of Amsterdam, The Netherlands	***Reporter***
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Invited Member

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ABSTRACT

Stress is a major risk factor for mood disorders, such as anxiety and depression. Rats exposed to prenatal restraint stress (PRS) - i.e. the offspring of dams submitted to repeated episodes of stress during the last 10 days of gestation - develop long-lasting biochemical and behavioral changes that recapitulate some traits of depression and anxiety. Mounting evidence suggests the involvement of hippocampal glutamatergic system in such disorders. Interestingly, the hippocampus represents an integral part of the altered programming triggered by PRS. Hence, we decided to investigate the glutamatergic hypothesis of depression in the rat model of PRS focusing on mechanisms of neuroadaptation within the hippocampal circuit. We found that PRS rats showed an impairment of glutamate release, in the ventral hippocampus, which is the specific portion of the hippocampus related to stress and emotions. Remarkably, local injections of drugs that enhanced glutamate release in the ventral hippocampus (i.e., a cocktail of GABA-B and mGlu2/3 receptor antagonists) had strong anxiolytic effects in PRS rats. In addition, chronic treatment with conventional antidepressant drugs enhanced glutamate release in the ventral hippocampus and corrected the anxious/depressive-like phenotype induced by PRS. Knowing that the ventral hippocampus modulates striatal motor programming, we extended the study of PRS rats to haloperidol-induced catalepsy, which models pharmacological parkinsonism in humans. We found that PRS rats were resistant to haloperidol-induced catalepsy as a result of an increased activity of motor thalamic nuclei, as assessed by stereologic counting of c-Fos-positive neurons. Our findings support the glutamatergic theory of stress-related mood disorders and suggest that an impairment of the ventral hippocampus and its influence on striatal circuit are key components of the neuroplastic program induced by PRS.

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THÈSE DE DOCTORAT

(Label Européen)

Soutenue le 15 Décembre 2012 par

Jordan MARROCCO

NOUVELLES AVANCÉES DANS L'HYPOTHÈSE GLUTAMATERGIQUE DE LA DÉPRESSION : UNE ÉTUDE NEUROCHIMIQUE ET PHARMACOLOGIQUE DANS LE MODÈLE DE STRESS PRÉNATAL CHEZ LE RAT

Membres du Jury

Dr Jean-Claude MICHALSKI	CNRS-Université de Lille 1, France	***Président du Jury***
Pr Anna PITTALUGA	University of Genoa, Italie	***Examinateur***
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RESUME

Le stress est un facteur de risque majeur pour les troubles de l'humeur comme l'anxiété et la dépression. Les rats exposés à un stress prénatal de contention (PRS) – *i.e.* la progéniture de mères soumises à des épisodes répétés de stress au cours des 10 derniers jours de gestation - développent des changements biochimiques et comportementaux durables qui résument certains traits de la dépression et de l'anxiété. Un nombre grandissant de travaux suggère l'implication du système glutamatergique hippocampique dans ces troubles. L'hippocampe fait partie intégrante de la programmation altérée déclenchée par le PRS. Nous avons donc décidé d'étudier l'hypothèse glutamatergique de la dépression chez le rat PRS, en mettant l'accent sur les mécanismes de neuroadaptation dans le circuit hippocampique. Nous avons démontré que les rats PRS présentaient une altération de la libération de glutamate dans l'hippocampe ventral, partie spécifique de l'hippocampe reliée au stress et aux émotions. Remarquablement, des injections locales de produits qui améliorent la libération du glutamate dans l'hippocampe ventral (*i.e.* un cocktail d'antagonistes des récepteurs au GABA-B et mGlu2/3) exerçaient un fort effet anxiolytique chez les rats PRS. De plus, un traitement chronique avec des antidépresseurs conventionnels améliorait la libération du glutamate dans l'hippocampe ventral et corrigeait le phénotype de type anxieux/dépressif induit par le PRS. Sachant que l'hippocampe ventral module la programmation motrice striatale, nous avons étendu notre étude chez le rat PRS à la catalepsie induite par l'halopéridol, qui modélise le parkinsonisme pharmacologique chez l'Homme. Nous avons constaté que les rats PRS étaient résistants à la catalepsie induite par l'halopéridol, comme le résultat d'une activité augmentée des noyaux thalamiques moteurs, et tel que révélé par le compte stéréologique des neurones c-Fos-positifs. Nos résultats renforcent la théorie glutamatergique dans les troubles de l'humeur liés au stress et suggèrent qu'une déficience de l'hippocampe ventral et son influence sur le circuit striatal sont des éléments clé du programme neuroplastique induit par le PRS.

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My heart leaps up when I behold

A rainbow in the sky:

So was it when my life began;

So is it now I am a man;

So be it when I shall grow old,

Or let me die!

The Child is father of the Man;

I could wish my days to be

Bound each to each by natural piety.

William Wordsworth, 1802

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GENERAL SUMMARY

The response to stress represents the ability to adapt and cope with diverse challenges of life events and tightly reflects the epigenetic programming of the individual. Accordingly, exposure to stressful events during critical periods of brain development triggers an epigenetic programming leading to low resilience to stress in the adult life. Stress, with the ensuing prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis, is a major risk factor for major psychiatric disorders, such as depression and anxiety. In addition, stress is known to induce consistent alterations of glutamatergic neurotransmission in brain regions involved in affect and emotions, such as the hippocampus, amygdala, and prefrontal cortex. Patients with recurrent depression, show reduced volume of the hippocampus and prefrontal cortex and altered patterns of activity in these brain regions on PET scanning and functional MRI. Thus, exposure to stress strongly affects some aspects of emotion and cognitive processing. There is increasing evidence suggesting the involvement of the glutamatergic system in mood and psychiatric disorders, such as major depression and anxiety. Several epidemiological studies on pregnant women have shown a high positive correlation between stressful events during pregnancy and dramatic effects at birth, such as spontaneous abortion, preterm birth, low body weight, developmental delays and long-term behavioral abnormalities. However, the investigation of the effects of early stress in humans results very difficult to perform, due to ethical concerns and retrospective approaches. Accordingly, during the last decades, specific animal models have been developed, especially in rats, where stress is applied to pregnant mothers or early in life, and the outcome is examined in the adult offspring. Rats exposed to prenatal restraint stress (PRS) - i.e. the offspring of dams submitted to repeated episodes of stress during the last 10 days of gestation - develop long-lasting biochemical and behavioral changes that likely reflect the induction of a pathological early programming. Alterations induced by PRS include a dysfunction of the HPA axis, a disorganization of circadian rhythms and the sleep-wake cycle, an age-dependent impairment in spatial learning, a lifelong reduction of hippocampal neurogenesis, and a long-term reduction in the expression and activity of group-I and group-II metabotropic glutamate (mGlu) receptors in the hippocampus. Remarkably, some of these changes are reversed by chronic antidepressant treatment. In addition, most of the abnormalities in synaptic transmission and plasticity in the hippocampus of PRS rats are found in the ventral hippocampus, the specific portion of the hippocampus that encodes memories related to stress and emotions. The PRS rat model meets the *criterium* of construct validity because it recapitulates environmental factors implicated in the etiology of depression and other stress-related disorders and, therefore, it represents a valid model for

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the investigation of new mechanisms that lie at the core of depression and for the design and screening of novel antidepressant drugs.

The primary objective of my PhD program **was to establish the role of hippocampal glutamate neurotransmission in the pathophysiology of the anxious/depressive profile induced by PRS.** With the supervision of my Tutors and the help of my colleagues **I attempted to evaluate the effect of PRS on glutamate transmission in the hippocampus and how these changes contribute to the pathophysiology of anxiety.** Then, **I examined the action of antidepressants on glutamate transmission** using agomelatine, a drug that behaves as a mixed MT1/MT2 melatonin receptor agonist and 5-HT2C receptor antagonist, and fluoxetine, a classical SSRI antidepressant.

In Chapter one, I report data on proteomic analysis in the hippocampus of adult male PRS rats, obtained by 2 dimensional electrophoresis and mass spectrometry **(see article #1)**. We found that PRS induced changes in the expression profile of a number of proteins involved in the regulation of signal transduction, synaptic vesicles, protein synthesis, cytoskeleton dynamics, and energetic metabolism. Interestingly, more than 20 percent of PRS-affected proteins were associated with neural plasticity and synaptic transmission.

In article #2, we extended our analysis to the glutamatergic synapse by measuring glutamate release in superfused synaptosomes and expression of vesicle-associated proteins, glutamate transporters, and glutamate receptors. We found that PRS rats showed a selective impairment of depolarization-stimulated glutamate and [3H]D-aspartate release in the ventral hippocampus with no changes in GABA release. Abnormalities of glutamate release were associated with large reductions in the levels of synaptic vesicle-related proteins, such as VAMP (synaptobrevin), syntaxin-1, synaptophysin, synapsin Ia/b and IIa, munc-18, and Rab3A in the ventral hippocampus of PRS rats. At least munc-12 and Rab3A are specifically associated to the glutamatergic synapse. As most of the alterations induced by PRS affect the ventral hippocampus and PRS rats show an anxious/depressive phenotype, we examined the relationship between glutamate release in the ventral hippocampus and anxiety. We found that anxiety-like behavior in male PRS (and control) rats was inversely related to the extent of depolarization-evoked glutamate release in the ventral hippocampus. We then demonstrated a causal relationship between anxiety-like behavior and reduction in glutamate release by using a cocktail of the mGlu2/3 receptor antagonist, LY341495, and the $GABA_B$ receptor antagonist, CGP52432. We showed that this cocktail could amplify depolarization-evoked [3H]D-aspartate release in the ventral hippocampus and that bilateral microinfusion of CGP52432 *plus* LY341495 in the ventral hippocampus totally abolished anxiety-like behavior in PRS rats **(see article #2)**.

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In Chapter two we examined whether chronic treatment with agomelatine and fluoxetine could modulate glutamate neurotransmission and anxiety, in an attempt to identify new molecular targets for antidepressant drugs. I wish to highlight that at least fluoxetine is widely used in the treatment of generalized anxiety disorder. We found that chronic treatment with agomelatine and fluoxetine reversed the anxious/depressive-like phenotype of PRS rats and corrected the abnormalities in glutamate release and in the expression of synaptic proteins in the hippocampus. The neuroproteomic phenotype of PRS rats was also corrected by antidepressant treatment **(see article #3)**. Thus, abnormalities of neural plasticity and glutamatergic transmission in the hippocampus represent an integral part of the altered programming triggered by early life stress.

In Chapter three, we moved from the evidence that toxin-induced damage of the ventral hippocampus restrains the motor response to antipsychotic drugs, suggesting that the ventral hippocampus modulates the basal ganglia motor circuit. Hence, we hypothesized that the abnormal glutamatergic transmission found in PRS rats could affect haloperidol-induced catalepsy, which models pharmacological parkinsonism in humans. We found that PRS were resistant to catalepsy induced by different doses of haloperidol. Reduction of catalepsy was likely due to an increased activity of ventral motor thalamic nuclei (VA, VL, and VM nuclei), which represent the terminal output stations of the basal ganglia motor circuit **(see article #4)**. The hypothesis that glutamatergic impairment in the ventral hippocampus may lie at the core of the resistance to haloperidol-induced catalepsy warrants further investigation.

Taken together, our results indicate that an impairment of glutamate release in the ventral hippocampus is a key component of the neuroplastic programming induced by PRS, and that strategies aimed at enhancing glutamate release in the ventral hippocampus correct the “anxious phenotype” caused by early life stress.

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RESUME GENERAL

La réponse au stress représente la capacité à s'adapter et faire face à diverses difficultés rencontrées au cours de la vie et est étroitement liée à la programmation épigénétique de l'individu. De fait, la confrontation à des événements anxiogènes durant les périodes critiques du développement cérébral induit une programmation épigénétique conduisant à une faible résilience au stress dans la vie adulte. Le stress, avec l'activation prolongée de l'axe hypothalamo-hypophyso-surrénalien (HPA) qui s'ensuit, est un facteur de risque majeur dans les troubles de l'humeur, tels que la dépression et l'anxiété. En outre, le stress est connu pour induire des altérations constantes de la neurotransmission du glutamate dans des régions du cerveau impliquées dans l'affect et les émotions, telles que l'hippocampe, l'amygdale et le cortex préfrontal. Chez les patients souffrant de dépression récurrente, on met en évidence une réduction du volume de l'hippocampe et du cortex préfrontal et une altération des *patterns* d'activité de ces régions en TEP et IRM fonctionnelle. Ainsi, l'exposition au stress affecte fortement certains aspects de l'émotion et de la cognition. Un nombre grandissant de travaux suggère l'implication du système glutamatergique dans les troubles de l'humeur et psychiatriques, comme la dépression majeure et l'anxiété. Plusieurs études épidémiologiques menées chez les femmes enceintes ont montré une forte corrélation positive entre les événements stressants pendant la grossesse et les effets dramatiques lors de la naissance, comme l'avortement spontané, l'accouchement prématuré, un faible poids corporel, des retards de développement, et des troubles du comportement à long terme. Toutefois, l'étude des effets du stress précoce chez l'Homme s'avère très difficile à réaliser, en raison de préoccupations d'ordre éthique et d'approches rétrospectives. En conséquence, au cours des dernières décennies, des modèles animaux spécifiques ont été développés, en particulier chez le rat, où le stress est appliqué à la mère gestante ou au début de la vie; et les conséquences sont examinées chez la progéniture adulte. Des rats exposés à un stress prénatal de contention (PRS pour Prenatal Restraint Stress) - c'est à dire la progéniture de femelles soumises à des épisodes répétés de stress au cours des 10 derniers jours de gestation – développent des changements biochimiques et comportementaux durables qui reflètent l'induction d'une programmation pathologique précoce. Les altérations induites par le PRS incluent un dysfonctionnement de l'axe HPA, une désorganisation des rythmes circadiens et du cycle veille-sommeil, un déficit de mémoire spatiale lié à l'âge, une réduction à vie de la neurogénèse hippocampique et une réduction à long terme de l'expression et de l'activité des récepteurs métabotropiques au glutamate du groupe I et du groupe II dans l'hippocampe.

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Remarquablement, certains de ces changements sont inversés par un traitement chronique aux antidépresseurs.

De plus, la plupart des anomalies de transmission synaptique et de plasticité dans l'hippocampe de rats PRS sont mises en évidence dans l'hippocampe ventral, la partie spécifique de l'hippocampe qui code la mémoire liée au stress et aux émotions. Le modèle de rat PRS répond au critère de validité conceptuelle (*construct validity*) car il récapitule les facteurs environnementaux impliqués dans l'étiologie de la dépression et d'autres troubles liés au stress et, par conséquent, il représente un modèle valide pour l'étude de nouveaux mécanismes qui sont au cœur de la dépression et pour la conception et le criblage de nouveaux traitements antidépresseurs.

Le premier objectif de mon programme de thèse était **d'établir le rôle de la neurotransmission hippocampique du glutamate dans la physiopathologie du profil anxieux/dépressif induit par le PRS.** Avec la supervision de mes Tuteurs et l'aide de mes collègues, **j'ai voulu évaluer l'effet du PRS sur la transmission du glutamate dans l'hippocampe et comment ces changements contribuaient à la physiopathologie de l'anxiété**. Ensuite, **j'ai examiné l'action d'antidépresseurs sur la transmission du glutamate** en utilisant l'agomélatine, un antidépresseur qui se comporte comme un agoniste mixte des récepteurs à la mélatonine MT1/MT2 et antagoniste des récepteurs 5-HT2C, ainsi que la fluoxétine, un antidépresseur ISRS classique.

Dans le chapitre un, je reporte les données de l'analyse protéomique dans l'hippocampe de rats PRS mâles adultes, obtenues par électrophorèse bidimensionnelle et spectrométrie de masse **(voir l'article #1)**. Nous avons montré que le PRS induisait des changements dans le profil d'expression d'un certain nombre de protéines impliquées dans la régulation de la transduction du signal, les vésicules synaptiques, la synthèse des protéines, la dynamique du cytosquelette et le métabolisme énergétique. De manière intéressante, plus de 20 % des protéines affectées par le PRS étaient associées à la plasticité neuronale et à la transmission synaptique.

Dans l'article #2, nous avons étendu notre analyse à la synapse glutamatergique en mesurant la libération de glutamate dans les synaptosomes superfusés et l'expression de protéines associées aux vésicules, de transporteurs du glutamate et de récepteurs au glutamate. Nous avons constaté que les rats PRS présentaient une déficience sélective dans la libération induite par la dépolarisation de glutamate et de [3H] D-aspartate dans l'hippocampe ventral avec aucun changement dans la libération du GABA. Les anomalies de la libération de glutamate étaient associées à des réductions importantes des niveaux de protéines liées à des vésicules synaptiques telles que VAMP (synaptobrévine), syntaxine-1, synaptophysine, synapsine Ia/b

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et IIa, munc-18, et Rab3A dans l'hippocampe ventral des rats PRS. Ainsi, munc-12 et Rab3A sont spécifiquement associées à la synapse glutamatergique. Dans la mesure où la plupart des altérations induites par le PRS affectent l'hippocampe ventral et comme les rats PRS présentent un phénotype anxieux/dépressif, nous avons examiné le lien entre la libération de glutamate dans l'hippocampe ventral et l'anxiété. Nous avons constaté que le comportement de type anxieux chez les rats mâles PRS (et les contrôles) était inversement relié à la libération de glutamate dans l'hippocampe ventral. Nous avons ensuite démontré une relation causale entre le comportement de type anxieux et la réduction de la libération de glutamate à l'aide d'un cocktail de l'antagoniste du récepteur mGlu2/3, LY341495, et de l'antagoniste du récepteur $GABA_B$, CGP52432. Nous avons montré que ce cocktail pouvait amplifier la libération de [3H] D-aspartate induite par la dépolarisation dans l'hippocampe ventral et que la microinfusion bilatérale de CGP52432 et de LY341495 dans l'hippocampe ventral abolissait totalement l'anxiété des rats PRS **(voir l'article #2)**.

Dans le chapitre deux, nous avons évalué si un traitement chronique avec l'agomélatine ou la fluoxétine pouvait moduler la neurotransmission glutamatergique et l'anxiété, dans le but d'identifier de nouvelles cibles moléculaires pour les antidépresseurs. Je souhaite souligner le fait que la fluoxétine est très utilisée dans le traitement de troubles anxieux généralisés.

Nous avons montré que le traitement chronique avec l'agomélatine et la fluoxétine inversait le phénotype de type anxieux/dépressif des rats PRS, et corrigeait les anomalies de libération de glutamate et d'expression de protéines synaptiques dans l'hippocampe. Le phénotype neuroprotéomique des rats PRS était également corrigé par un traitement avec des antidépresseurs **(voir l'article #3)**. Ainsi, des anomalies de plasticité neuronale et de transmission glutamatergique dans l'hippocampe représentent une partie intégrale de la programmation altérée déclenchée par un stress précoce.

Au chapitre trois, nous sommes partis de l'évidence que les dommages de l'hippocampe ventral induits par les toxines conditionnent la réponse motrice aux antipsychotiques, suggérant que l'hippocampe ventral module le circuit moteur des ganglions de la base. Par conséquence, nous avons émis l'hypothèse que la transmission glutamatergique anormale mise en évidence chez les rats PRS pourrait affecter la catalepsie induite par l'halopéridol, qui modélise le parkinsonisme pharmacologique chez l'humain. Nous avons constaté que les PRS étaient résistants à la catalepsie induite par différentes doses d'halopéridol.

La réduction de la catalepsie était due à une activité augmentée des noyaux thalamiques moteurs ventraux (noyaux VA, VL, et VM), qui représentent les stations terminales du circuit moteur des ganglions de la base **(voir l'article #4)**. L'hypothèse selon laquelle l'altération glutamatergique dans l'hippocampe ventral peut être à la base de la résistance à la catalepsie

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induite par l'halopéridol nécessite expériences ultérieures. Considérés dans leur ensemble, nos résultats indiquent qu'une altération de la libération de glutamate dans l'hippocampe ventral est un élément clé du programme neuroplastique induit par le PRS, et que les stratégies visant à améliorer la libération de glutamate dans l'hippocampe ventral corrigent le «phénotype anxieux» causé par le stress précoce.

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INTRODUCTION

1 Early programming of stress-related adult disease

1.1 Stress and circuitry of the stress system

Habib and collaborators (2001) stated that life is a dynamic and complex equilibrium that is maintained by a continuous challenge towards intrinsic and extrinsic strength: the stressors. A "stressor" is an event or experience that threatens the ability of an individual to adapt and cope with life events (Lazarus and Folkman, 1984). The diverse responses to stressors implicate the sensitiveness and the programming of the genetic and epigenetic profile of the individual (see below). The idea of *"milieu intérieur"* was introduced for the first time by Claude Bernard (1878), who proposed that the internal environment is constant even though the external environmental conditions change. In 1932, Walther Cannon called this process "homeostasis", from the Greek *homo* (the same) and *stasis* (to stay) and he studied for the first time physiological variations in response to environmental stimuli (Cannon, 1932). Cannon proposed that unpredictable stimuli could activate a stress machinery that includes endocrine and immune responses and that involve the entire central and peripheral nervous system. The term "stress" was defined for the first time by Hans Selye in 1935. Stress is a "multidimensional concept" that includes three main components:

1. The stimulus (stressor) that is negative or positive.
2. The cognitive evaluation of the stressor. It depends on life experiences and ability to predict stressful events.
3. Individual physiological responses.

According to Selye, physiological responses are organized in three phases (Selye, 1976). First, the general alarm reaction, during which lots of biological systems, including the hypothalamic-pituitary-adrenal axis (HPA), is activated. The second phase consists in contrasting the changes by feedback messages that signal to return to the "normal" state. Lastly, if the stressful event is maintained, the system loses any possibility of resistance and enters in an exhaustive phase.

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Stress	Activation of a stress response, a stressful stimulus itself, and/or the consequences of a stressful experience.
Stressor	Stimulus or event that challenges the organism with a potential threat and that induces a physiological and behavioral response. Unpredictability is a reinforcing factor, and a stressor that is unexpected, cannot be controlled or avoided, and has uncertain consequences is more severe. Unpredictable stressors are distinguished from stimuli that vary expectedly such as seasonal shifts that homeostatically increase stress axis activity.
Stress response	Mobilizes resources from metabolic, cardiovascular, autonomic, immune, and CNS to adapt to stimulus. Comprised of activation, recovery and adaptation. Rapid activation by stress is followed by quick recovery, except after traumatic events (e.g., combat, life-threatening accident, assault). Such events can lead to chronic stress that engages long-term adaptive coping mechanisms. These mechanisms can become maladaptive and increase vulnerability to stress-related psychophathology (e.g., PTSD).
Basic stress physiology	Stress hormones and neuropeptides include CRH, urocortins, ACTH, glucocorticoids, vasopressin, endorphins, and neurotransmitters such as adrenaline. CRH acts through CRH receptor 1 (CRHR1) and to a lesser extent CRHR2 (Refojo and Holsboer, 2009). Urocortin 2 and 3, CRH-related peptides, preferentially bind to CRHR2. CRHR1 is predominant in PFC, hippocampus, PVN, anterior pituitary, and BLA. CRHR2 is abundant in the ventromedial hypothalamus, DRN, and medial amygdala (Steckler and Holsboer, 1999). CRH-CRHR1 is involved in stress response initiation and urocortin-CRHR2 system in termination. CRHRs are linked to intracellular Gs/adenylyl cyclase-dependent and MAPK signaling.
Actions of glucocorticoids	Upon entering the brain, glucocorticoids (GC) rapidly increase stress reactions, then contain these reactions to facilitate recovery, while promoting behavioral adaptation. Moderate and controlled GC levels mediate normal cellular functions, while low or excessively high levels cause dysfunction. GCs act via mineralocorticoid (MR) and glucocorticoid (GR) receptors. MRs are involved in stress appraisal and response onset, while GRs facilitate response termination and the establishment of coping strategies. MR/GR imbalance is associated with stress vulnerability and related pathologies (De Kloet et al., 1998). Chronically elevated GC compromise neuronal survival and plasticity, neurotrophic factor expression, chemoresistance to oxidative stress, and promote inflammatory cascades.

Table 1: Summary of key-words and significance commonly employed to study stress
(from Franklin *et al.*, 2012)

A central construct in Seyle's integrative model of stress was the notion of homeostasis, which is the return to the *status quo ante.* More recently, McEwen and coworkers applied the concept of homeostasis only to a limited number of physiologic variables (end points), such as pH, body temperature, glucose levels and oxygen tension, which are essential for life and are maintained within a narrow range of their respective set-points. These set-points and other boundaries of control may themselves change with environmental conditions; however, these changes cannot be explained solely by the notion of homeostasis. The terms “allostasis” was therefore introduced to suggest that regulation is not constant but it adapts to brain plasticity (Sterling and Eyer, 1988). Accordingly, it has been proposed that an “allostatic overload” would lead to the reduction in neural plasticity and, as a consequence, to a higher vulnerability to psychiatric and cardiovascular diseases. Thus, in the new terminology of allostasis, Selye's alarm response is reinterpreted as the process leading to adaptation, or allostasis, in which stress hormones promote adaptation to the stressor. Selye's stage of resistance reflects the protective effects of the adaptation to the stressor. But if the alarm response is sustained and the neurochemical impairment is maintained over many days, an allostatic state may ensue, leading to allostatic overload, which replaces Selye's stage of exhaustion. Here, it is important to highlight that this state represents the almost inevitable wear and tear produced by repeated exposure to mediators of allostasis, i.e., too much of a good thing. Thus, Selye's diseases of adaptation are the result of the allostatic state leading to

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allostatic overload and resulting in the exacerbation of pathophysiologic changes (McEwen, 2005).

Activation of the HPA axis is the main target of the stress response. Two neuropeptides, corticotropin-releasing hormone (CRH) and vasopressin (AVP), are essential for coordinating the behavioural and metabolic responses to stress (Fig. 1). The hypothalamic release of CRH and vasopressin govern the HPA axis, the activity of which is reflected by blood concentrations of corticosteroid hormones that act through the modulation of gene transcription (de Kloet *et al.*, 2005). The receptor system that mediates the slow genomic actions of corticosteroids consists of two related receptor molecules, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), which bind the same hormone (primarily cortisol in humans and corticosterone in rodents) in the brain, albeit with a tenfold difference in affinity (Reul and de Kloet, 1985; Arriza *et al.*, 1988). MR affinity seems sufficiently high to maintain receptor activation throughout 1-h intervals between hormone secretory bursts of 20-min duration. By contrast, the lower affinity GR seems to respond largely in phase with the ultradian rhythm. This receptor becomes progressively activated during stress- and circadian-induced increases in the frequency and amplitude of corticosteroid secretory bursts (Young *et al.*, 2004; Kitchener *et al.*, 2004). Below is a more accurate description of the HPA-stress axis, as well as the mediators and structures implicated in the response to stress.

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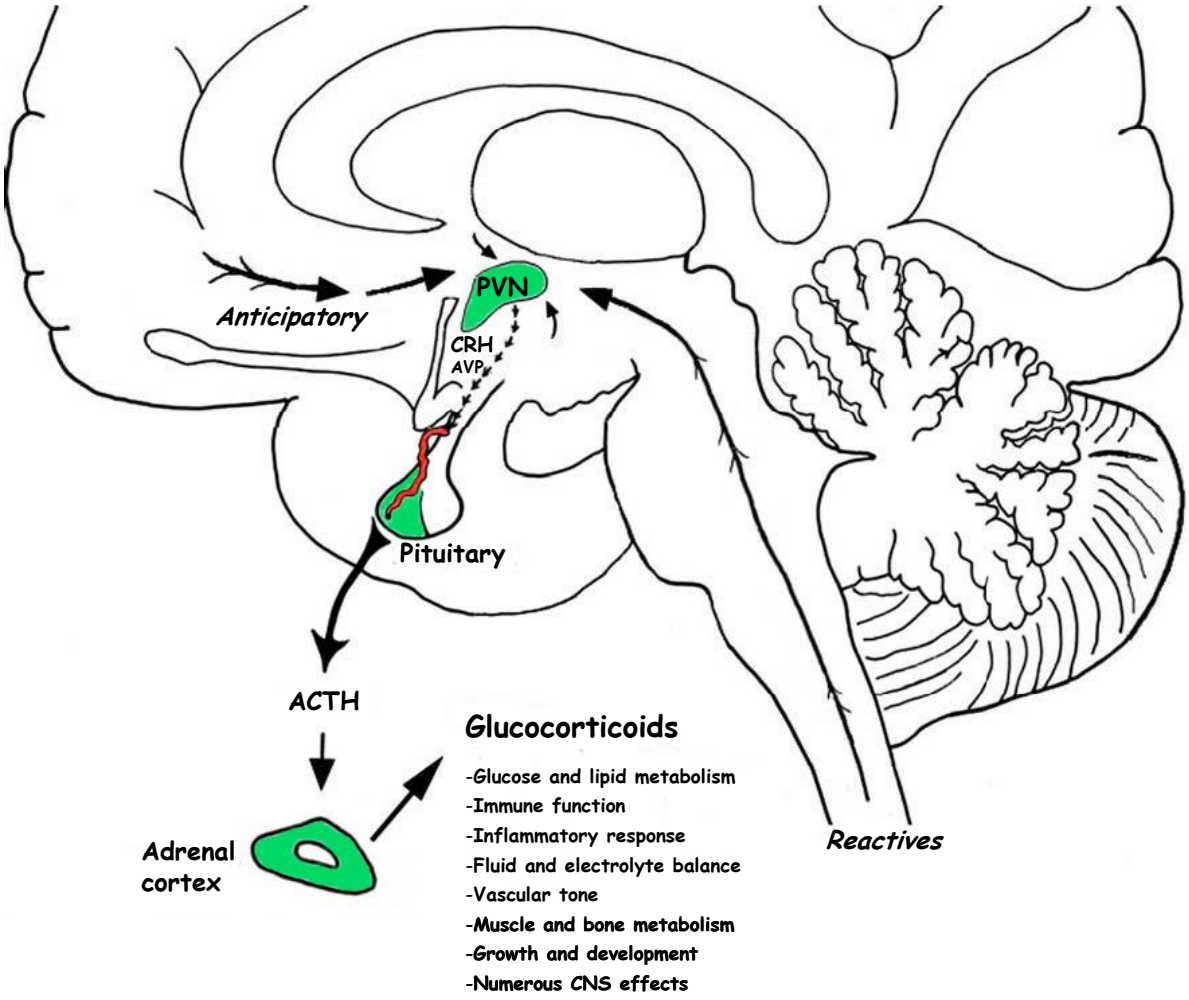

Figure 1: Overview of the hypothalamic–pituitary–adrenocortical axis, including principal classes of regulatory afferents and corticosteroid actions. CRH (corticotropin releasing hormone) and AVP (arginin vasopressin) neurons located within the medial hypothalamic paraventricular nucleus (PVN) drive pituitary corticotrophs via the portal vasculature, stimulating the release of ACTH. ACTH, in turn, mediates the synthesis and release of corticosteroids from the adrenals. CRH neurons are regulated by sensory afferents which are relayed via brainstem loci, and transmit ‘‘reactive” stimuli which are generally excitatory and relatively direct. Conversely, limbic forebrain structures are hypothesized to convey ‘‘anticipatory’’ signals that involve processing within pathways proximal to the level of the PVN, including in the peri-PVN area and several local hypothalamic regions. Integration of ‘‘anticipatory” circuits and neural pathways subserving “reactive” responses occurs at multiple levels (not shown on this figure, see the following text). (adapted from de Kloet *et al.*, 2005).

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1.1.1 Neural circuits and HPA-stressed axis

The HPA axis is controlled by a discrete pool of hypophysiotrophic neurons located in the medial parvocellulaire division of the paraventriculaire nucleus of hypothalamus (mpPVN) (Fig. 1) (Antoni, 1986; Whitnall, 1993). This small population of neurons (approximately 4000 in the rat (Swanson, 1987) synthesizes and releases the CRH, the principal secretagogue of the adrenocorticotropic hormone (ACTH). CRH mediates ACTH release under basal and stressful conditions (Antoni, 1986; Whitnall, 1993). The most important peptide produced by CRH-cells, which acts synergistically with CRH to increase the "gain" of the ACTH response (Antoni, 1986; Lowry *et al.*, 1986; Whitnall, 1993). CRH and AVP reach the corticotropic cells of the anterior pituitary *via* the hypothalamo-hypophyseal portal capillary network running on the ventral face of median eminence. In the anterior pituitary, CRH and AVP stimulate the synthesis and release of ACTH, which is the cleavage product of proopiomelanocortine (POMC). CRH activates type-1 CRH receptors coupled to Gs proteins, whereas AVP activates V1b receptors coupled to Gq proteins (Van Pett *et al.*, 2000). ACTH, a peptide of 34 amino acids carrying its biological activity in its 24 N-terminal amino acids, is released into the systemic circulation and acts on the fasciculate and reticulate portions of the adrenal cortex to stimulate the production and release of GCs and androgens. ACTH can also stimulate the production of mineralcorticoids in the glomerular portion of the adrenal cortex, although blood potassium concentrations and angiotensin-II are the major physiological stimuli for mineralcorticoid production (Antoni, 1986; Whitnall, 1993). In the adrenal cortex, ACTH activates type-2 melanocortin receptors coupled to Gs proteins. Other hormones, cytokines, and neuronal information from the autonomic nerves of the adrenal cortex are also involved in the regulation of GC secretion (Charmandari *et al.*, 2005).

Under non-stressful conditions, both CRH and AVP are secreted in the portal system in a circadian, pulsatile, and highly concordant fashion. The amplitude of the CRH and AVP pulses increases at the end of the light period (in the rat), resulting in increases in the amplitude of pulsate of secretion of ACTH and GCs at the beginning of the active phase of the diurnal cycle primarily in the amplitude of the pulsatile ACTH and GC secretion (Keller-Wood and Dallman, 1984). Diurnal variations in the pulsatile secretion of ACTH and GCs are often perturbed by changes in lighting, feeding schedules, and activity, as well as by stress. In addition, depending on the stressor, other factors, such as angiotensin II, various cytokines, and lipid mediators of inflammation are secreted and act on the hypothalamic, pituitary, and/or adrenal components of the HPA axis and potentiate its activity (Charmandari *et al.*, 2005).

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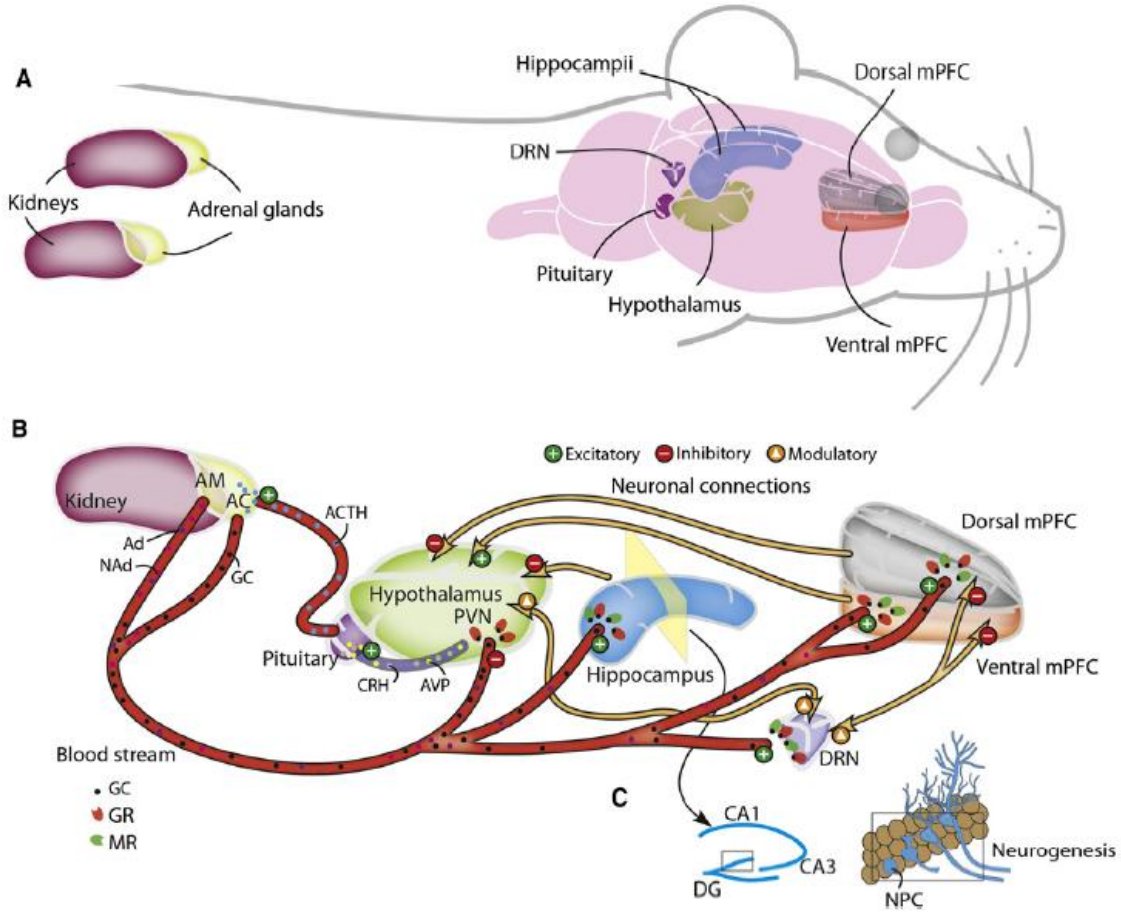

Figure 2: (A) Major components of the HPA axis and connected brain structures. (B) Connections between the hypothalamus, pituitary, and adrenal glands in the HPA axis, and hippocampus, medial prefrontal cortex (mPFC), and DRN. Activation of the HPA axis is initiated by stimulation of neurons in the medial parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus and secretion of corticotropin-releasing hormone (CRH, or corticotropin-releasing factor, CRF) and arginine vasopressin (AVP) that amplifies the effect of CRH, in the portal vein. The pituitary gland secretes adrenocorticotropic hormone (ACTH), initiating the release of glucocorticoids (GC) from the adrenal cortex (AC), and adrenaline (Ad) and noradrenaline (NAd) from the adrenal medulla (AM) into the blood stream. This cascade is transient, and upon termination or removal of the stimulus, the HPA axis returns to a baseline state by the action of several negative feedback loops. In these loops, GC act directly to shut down the response of the hypothalamus and pituitary, and the release of CRH then ACTH, and indirectly by activating glucocorticoid receptors (GRs) in the hippocampus and frontal cortex, that project back to the hypothalamus. GC also activate mineralocorticoid receptors (MRs). The hypothalamus and mPFC have reciprocal projections with the dorsal raphe nucleus (DRN). Neurogenesis occurs in the dentate gyrus and yields new neurons from neural progenitor cells (NPCs). (From Franklin *et al.*, 2012).

a) The peri-PVN area

CRH neurons of the mpPVN receive very few direct afferent fibers from the limbic system. The majority of these afferent inputs are relayed by glutamatergic (Ziegler and Herman, 2000) and GABAergic interneurons localized within the immediate environment of the PVN, which

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can be named the peri-PVN area, and within the intermediate layer (see the next point). CRH neurons controlling the HPA activity largely express glutamate and GABA receptors. Thus, activation of these CRH neurons may occur in response to both glutamatergic stimulation and inhibition of GABAergic interneurons projecting to CRH neurons. In contrast, inhibition of CRH neurons ensues from the activation of GABAergic interneurons. (Herman *et al.*, 2002)

b) Intermediate areas

The bed nucleus of the stria terminalis, the ventrolateral preoptic area, and the dorsomedial hypothalamic nucleus sends a rich GABAergic innervation to CRH neurons of the mpPVN. These GABAergics neurons gather the afferent inputs originating from structures of the "third level", such as the hippocampus, amygdala and prefrontal cortex. The BNST can be subdivided into two sub-regions, which exert an opposite regulation on the HPA axis. The anterior and posterior portion of the BNST activates and inhibits mpPVN CRH neurons, respectively (Herman *et al.*, 2003).

The subfornical body, the area postrema and the oganum vasculosum of the lamina terminalis are very sensitive to hydrous and osmotic balances. These regions, in which the blood-brain barrier is permeable, send direct projections to CRH neurons and can activate the HPA axis in response to hemorrhagic, osmotic or auditory stress (Herman *et al.*, 2003).

c) Limbic areas and noradrenergic centers of the brainstem

These areas receive sensory-motor afferent inputs originating from the cerebral cortex as well as from the peripheral sensory system. Noradrenergic centers of the brainstem such as the locus coeruleus (LC) and the nucleus of the solitary tract (NST), send direct noradrenergic fibers to mpPVN CRH neurons. The hippocampus, prefrontal cortex and amygdala exert an important control on the HPA axis, which is largely mediated by GABAergic neurons projecting to the mpPVN (Herman *et al.*, 2003, 2005; de Kloet *et al.*, 2005).

Noradrenergic centers of the brain stem

Noradrenaline reinforces the inhibitory activity of GABAergic neurons on the HPA axis. In the BNST, noradrenaline decreases glutamate release from hippocampal nerve endings, thereby restraining the inhibitory control of the hippocampus on the mpNVP (for review: Forray and Gysling, 2004). Noradrenaline can also directly activate GABAergic neurons that exert an inhicitory control on the HPA axis (Han *et al.*, 2002). The stimulatory and inhibitory actions of noradrenaline are mediated by α1 and α2 receptors, respectively (Plotksy *et al.*,

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1989). Glutamatergic neurons of the peri-PVN region are also regulated by noradrenaline, which, in this particular case, activates the HPA axis (Herman *et al.*, 2003). Finally, noradrenaline can directly modulate CRH neurons, which express both α1 (excitatory) and β (inhibitory) adrenergic receptors (Daftary *et al.*, 2000).

The hippocampus

Many studies related to hippocampus stimulations and lesions are in agreement to give to this structure a major inhibitory function on the HPA axis. Indeed stimulation of the hippocampus induces a reduction in the release of GCs whereas its lesion increases the activation of the HPA axis both in basal condition and after a stress (Dunn and Orr, 1984; Herman *et al.*, 1989). This inhibitory function could be particularly important during the return at a basal level of activation of the HPA axis following its activation by stress. This function in the HPA axis negative feedback implies preferentially the subiculum of the ventral hippocampus (Herman *et al.*, 1995). However the hippocampus does not emit direct projection to the PVN. These inhibitory effects involve the rich hippocampal glutamatergic innervation projecting to the GABAergic neurons which contacting directly the mpPVN CRH neurons.

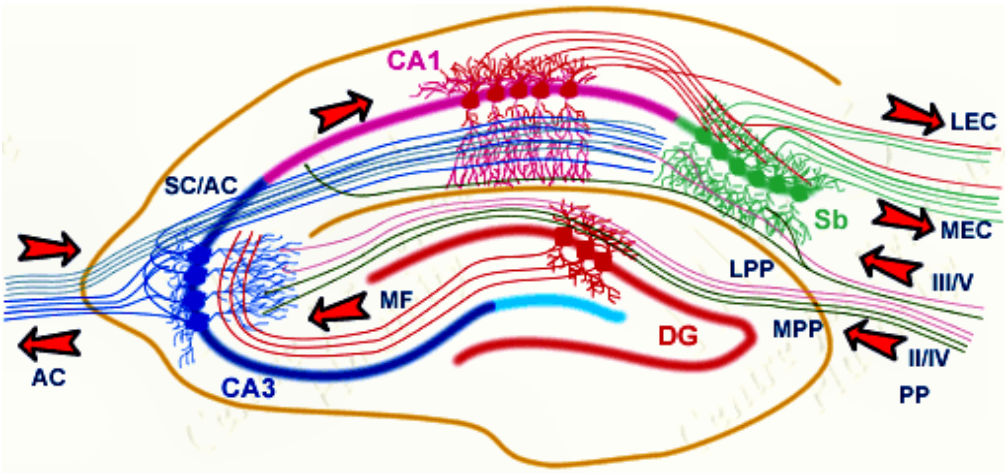

Figure 3: The hippocampal network. The hippocampus forms principally an unidirectional network. The inputs coming from the entorhinal cortex (EC) connect the granular neurons of the Dentate gyrus (DG) and the pyramidal neurons of the CA3 area via the lateral and medial perforant path (LPP and MPP, respectively). CA3 pyramidal neurons receive also inputs from the DG via the mossy fibers (MF). These neurones send theirs axons to the pyramidal neurons of the CA1 area via the Schaffer collateral (SC) as well as to the pyramidal neurons of the CA1 area of the controlateral hippocampus via the anterior commissure (AC). Neurons of the CA1 receive also directly inputs from the perforant path and send their axone to the neurons of the subiculum (Sb). These neurons send in turn the principal efferent fibers of the hippocampus to the entorhinal cortex forming a loop.
Adapted from: *http://www.bris.ac.uk/Depts/Synaptic/info/pathway/hippocampal.ht*

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The medial prefrontal cortex

This structure is usually proposed to inhibit the HPA axis. It appears that this effect seems to be contrasted and regionally-specific. Whereas the prelimbic cortex and the anterior subiculum send many excitatory projections towards the BNST, and hypothalamic GABAergic neurons inhibiting the mpPVN CRH neurons, the infralimbic cortex projects mainly towards the medial and central nuclei of amygdala, the NTS and the anterior BNST. Contrary to the other subdivisions of the mprefrontal cortex, these projections of the infralimbic cortex are in a position being able to confer an excitatory role rather an inhibitory on the HPA axis (For review: Herman *et al.*, 2003, 2005).

The amygdala

The amygdala, via its medial part (MeA) and central nucleus (CeA), activates the HPA axis. Whereas MeA activates the HPA axis in response to emotional stress (forced swimming, predator, social interaction…) the CeA is also implied in the HPA response to inflammatory or hemodynamic stress. This role of amygdala goes together with its stimulatory functions on the autonomic nervous system as well as its implication in the control of the fear and anxiety. The CeA is strongly connected to noradrenergic centers of the brain stem like NTS and LC and can thus indirectly activate the HPA axis through these brainstem structures. CeA and MeA project also largely on the GABAergics neurons controlling the activity of the mpPVN CRH neurons. But, contrary to the hippocampal projections, the amygdala projections are GABAergics. Thus, the amygdala activation generates a disinhibition of the HPA axis by inhibiting the GABAergic projecting to the mpPVN CRH neurons. (Herman *et al.*, 2003, 2005)

1.1.2 Individual coping and stress-related disorders

There is high consensus that stress-related disorders are shaped by nature × nurture interactions. Particularly important in nature × nurture research is uncovering the mechanisms whereby nature (genes) influence disease risk as a function of nurture (environmental stimuli). However, research has led to contradictory data and the complexity of nature × nurture interactions appears larger than envisaged. This hampers the understanding of individual differences in vulnerability to stress-related disorders and their treatment. Mainstream research is governed by the Diathesis-Stress/Dual Risk hypothesis (Burmeister *et al.*, 2008; Sameroff and Seifer, 1983) that some individuals, because of a genetic "vulnerability", are disproportionately or even exclusively likely to be affected adversely by an environmental stressor. However, it is unlikely that these genes are maintained throughout

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evolution when they exert outright negative effects. Accordingly, the 'for-better-and-for-worse' (Belsky *et al.*, 2009) concept was introduced, which is based on the idea that 'stress-sensitive' genes actually are 'plasticity' genes. These plasticity genes turn out maladaptive in impoverished, aversive environments, and adaptive in favorable environments. Ellis and coworkers (2011) proposed the "biological sensitivity to context" hypothesis arguing that individuals vary in their susceptibility to environmental influences in much the same way as the "for-better-and-for-worse" concept for nature × nurture interactions, with the difference that they do not presume that this environment-driven variability is mediated by genotype. Rather, it is their view that experience can shape plasticity, and that a 'fit' between the person and his/her environment determines 'for-better-and-for-worse' outcomes. This evolutionary grounded view relates to the 'environmental mismatch' hypothesis recently proposed by Mathias Schmidt (2011), postulating that depression might be promoted by a mismatch of the programmed and the later actual environment in combination with a more vulnerable or resilient genetic predisposition. Because the 'environmental fit' has much to do with how we cope with environmental challenges, Homberg introduced the 'stress-coping (mis)match (SCM)' hypothesis, which postulates that stress-coping responses—as programmed by nature × nurture interactions—are adaptive when they match current stress conditions, but maladaptive when they mismatch them (Homberg, 2012).

Although there is very little evidence regarding the effects of ordinary life stressors on brain structure, there are indications from functional imaging of individuals undergoing ordinary stressors, such as counting backwards that there are lasting changes in neural activity (Wang *et al.*, 2005). Another study, using voxel-based morphometry, has uncovered a relationship between shrinkage of grey matter volume in the hippocampus and orbitofrontal cortex and prospective reports of chronic life stress over a 20 year period (Gianaros *et al.*, 2007). Moreover, the study of depressive illness and anxiety disorders has also provided a number of insights. For example, life events are known to precipitate depressive illness in individuals with certain genetic predispositions (Kessler, 1997; Kendler, 1998; Caspi *et al.*, 2003). Moreover, brain regions such as the hippocampus, amygdala and prefrontal cortex show altered patterns of activity in PET (positron emission tomography) and fMRI (functional magnetic resonance imaging) and also demonstrate changes in volume of these structures in individuals with recurrent depression: decreased volume of hippocampus, prefrontal cortex and amygdala (Drevets *et al.*, 1997; Sheline *et al.*, 1999, 2003). Interestingly, amygdala volume has been reported to increase in the first episode of depression, whereas hippocampal volume is not decreased (MacQueen *et al.*, 2003; Frodl *et al.*, 2003). It has been known for some time that stress hormones, such as cortisol, are involved in psychopathology, reflecting

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emotional arousal and psychic disorganization rather than the specific disorder *per se* (Sachar *et al.*, 1973). We now know that adrenocortical hormones enter the brain and produce a wide range of effects upon it. In Cushing's disease, there are depressive symptoms that can be relieved by surgical correction of the hypercortisolemia (Starkman and Schteingart, 1981; Murphy, 1991). Both major depression and Cushing's disease are associated with chronic elevation of cortisol that results in gradual loss of minerals from bone and abdominal obesity. In major depressive illness, as well as in Cushing's disease, the duration of the illness and not the age of the subjects predicts a progressive reduction in volume of the hippocampus, determined by structural magnetic resonance imaging (Starkman *et al.*, 1992; Sheline *et al.*, 1999). Moreover, there are a variety of other anxiety-related disorders, such as posttraumatic stress disorder (PTSD) (Bremner, 2002; Pitman, 2001) and borderline personality disorder (Driessen *et al.*, 2000), in which atrophy of the hippocampus has been reported, suggesting that this is a common process reflecting chronic imbalance in the activity of adaptive systems, such as the HPA axis, but also including endogenous neurotransmitters, such as glutamate (see below). Also, The changes of tissue sensitivity to glucocorticoids are associated with many pathological states including neurological diseases. There is strong evidence that Alzheimer's disease is linked to abnormal functions of glucocorticoids, which is reflected not only in the changes of activation of the HPA axis but also in clinical syndromes (Dai *et al.*, 2004). Another important factor in hippocampal volume and function is glucose regulation. Outright Type 2 diabetes and poor glucose control as measured by glycosylated hemoglobin is associated with reduced hippocampal volume (Gold *et al.*, 2007). Furthermore, poor glucose regulation is associated with smaller hippocampal volume and poorer memory function in individuals in their 60s and 70s who have "mild cognitive impairment" (Convit, 2005), and both mild cognitive impairment and Type 2, as well as Type 1, diabetes are recognized as risk factors for dementia (Ott *et al.*, 1996; de Leon *et al.*, 2001; Haan, 2006). Having a positive outlook on life and good self-esteem appear to have long-lasting health consequences (Pressman and Cohen, 2005), and good social support is also a positive influence on the measures of allostatic load (Seeman *et al.*, 2002). Positive affect, assessed by aggregating momentary experiences throughout a working or leisure day, was found to be associated with lower cortisol production and higher heart rate variability (showing higher parasympathetic activity), as well as a lower fibrinogen response to a mental stress test (Steptoe *et al.*, 2005). On the other hand, poor self-esteem has been shown to cause recurrent increases in cortisol levels during a repetition of a public speaking challenge in which those individuals with good self-esteem are able to habituate, i.e., attenuate their cortisol response after the first speech (Kirschbaum *et al.*, 1995). Furthermore, poor self-esteem and low

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internal locus of control have been related to 12–13% smaller volume of the hippocampus, as well as higher cortisol levels during a mental arithmetic stressor (Pruessner *et al.*, 1999, 2005). Related to both positive affect and self-esteem is the role of friends and social interactions in maintaining a healthy outlook on life. Loneliness, often found in people with low self-esteem, has been associated with larger cortisol responses to wakening in the morning, and higher fibrinogen and natural killer cell responses to a mental stress test, as well as sleep problems (Steptoe *et al.*, 2004). On the other hand, having 3 or more regular social contacts, as opposed to 0 to 2 such contacts, is associated with lower allostatic load scores (Seeman *et al.*, 2002).

1.1.3 Stress-sensitive pathways

The limbic structures that we have described above (hippocampus, amygdala, and prefrontal cortex) take a fundamental part in the control of the HPA axis activity and they are also implicated in cognitive processes such as memory, behavioral reactivity to the novelty and anxiety.

Several data during this last thirty years suggest that the neuronal circuits and their connections are prone to many modifications and reorganizations throughout the life. The processes by which the brain adapts its function to the internal and external stimuli are now classically indicated like neuronal processes of plasticity. These dynamic processes are based on the abilities of the neuronal systems, the brain nuclei, the neurons, the synapses, the receptors and up to the transcription factors, to change their structural and functional repertoire in response to alterations of the internal and/or external environment (Zilles, 1992).

In adulthood, the limbic system presents two large levels of plasticity. First, the synaptic and dendritic plasticity in the Ammon horns (CA) as well as in the dentate gyrus (GD) of the hippocampus, the amygdala nuclei and the prefrontal cortex; second, the plasticity may result from the production of new cells in particular in the GD of the hippocampus. Neurogenesis is the ability of specific regions of the adult brain to produce new cells (neurons, astrocytes) able to be integrated into the pre-existing neural networks. Adult neurogenesis takes place in an intense manner in two cerebral areas: the sub-ventricular zone bordering the lateral ventricles and the hippocampus (Rakic, 2002; Alvarez-Buylla and Garcia-Verdugo, 2002). Several studies showed that this cytogenesis and in particular the hippocampal neurogenesis was modulated by the environmental factors such as novelty and stress. These observations suggested that the hippocampal neurogenesis is not only a process of passive replacement of the lost cells, but mostly an adaptive response to the

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modifications of the internal and/or external environment. Also, chronic stress affects dendritic complexity and synaptic function in rat hippocampus (Krugers *et al.*, 2010; Joëls 2008; Joëls *et al.*, 2004) whereas a single social stress-experience alters glutamate receptor-binding in rat hippocampal CA3 area (Krugers *et al.*, 1993). Thus, synapses have the ability to change their strength in response to environmental stimuli. This mechanism is known as long term potentiation (LTP) and it is considered one of the major cellular mechanisms that underlie learning and memory (Bliss and Collingridge, 1993; Cooke and Bliss, 2006). In particular, lesions within the ventral hippocampus but not the dorsal hippocampus affect the anxiety-like behaviors (Pentkwoski *et al.*, 2006). The dorsal hippocampus remains strongly implied in the processes of learning. The neural connection with amygdala combined to a high expression of 5-HT 2c receptors, suggest that hippocampus is a key structure in mood disorders and anxiety modulation (Alves *et al.*, 2004). Synaptic plasticity was particularly well described at the level of the hippocampus with the phenomena of long-term potentiation and depressions (respectively LTP and LTD). These phenomena refer to the increases (LTP) or reductions (LTD) in the effectiveness of the synaptic transmission in time. It has been proposed that many stress- and anxiety-related disorders have a common basis represented by an excessive or inappropriate cerebral excitability associated to the expression of the symptoms (Swanson *et al.*, 2005). Stress play a central role in mediating LTP and LTD *via* the glutamate N-methyl-D-aspartate (NMDA) receptor, thus the modulation of the glutamatergic system is a key feature to assess synaptic plasticity and maintenance. However, the role of stress on LTP remains equivocal, as the direction of LTP would depend both on the type of stress and the brain area of investigation . Moreover, in some situations and in some individuals particular challenging situations may lead to more sympathetic drive relative to the HPA-axis or *vice versa*. As both noradrenaline and CRH increase LTP, the abundance of these hormones relative to that of corticosterone is very important in determining the overall effect of stress. Also, while it is generally agreed that LTP depending on NMDA-receptor activation is impaired by stress and corticosterone, such impairment is not always seen for other forms of LTP (Joëls and Krugers, 2007).

1.1.3.1 Stress, glutamate and plasticity

Glutamate is the major excitatory neurotransmitter in the brain but it is also an intermediary metabolite in the detoxification of ammonia and an important building block for the synthesis of peptides and proteins. The high concentration of glutamate within the cells of the CNS requires a fine regulation process to ensure optimal neurotransmission and prevent excitotoxicicty (Fig. 4).

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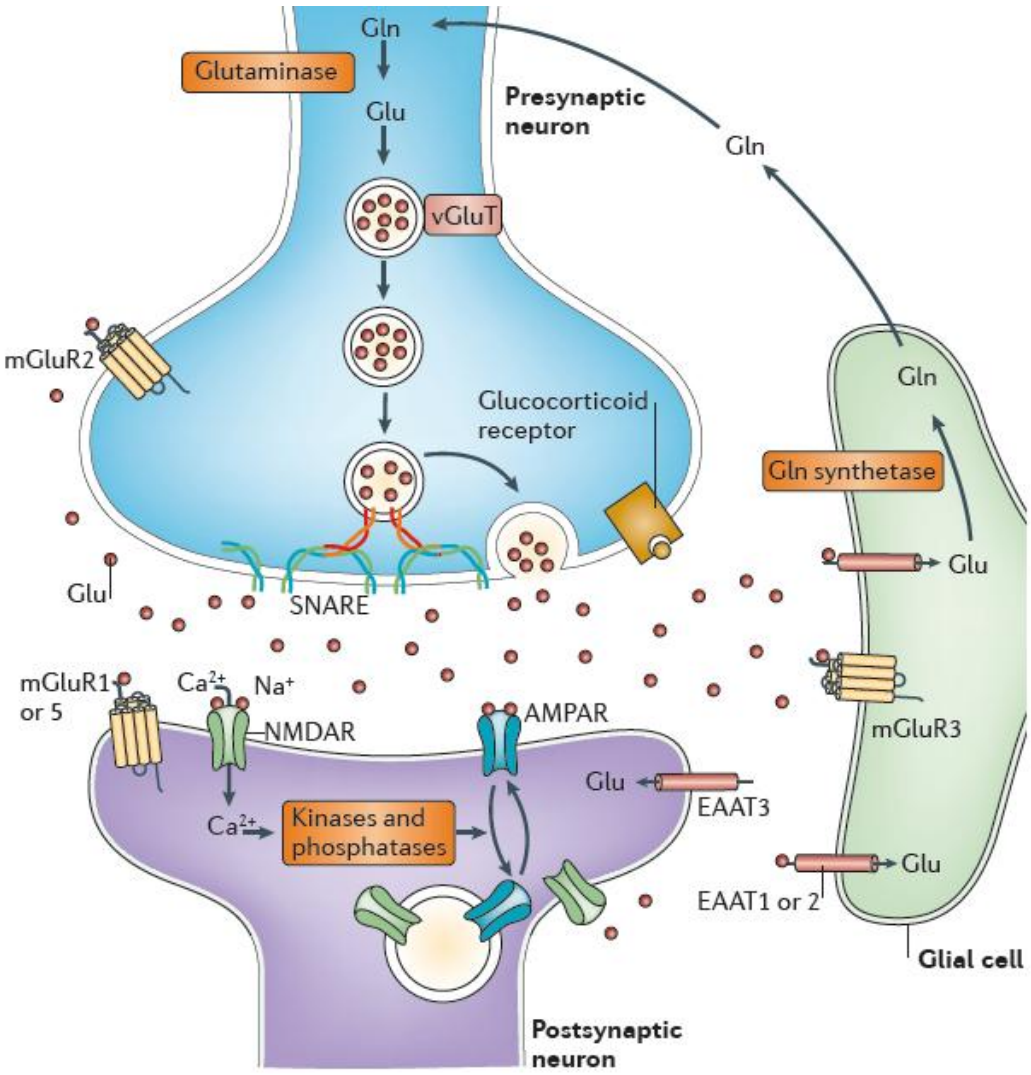

Figure 4: The glutamate tripartite synapse. Neuronal glutamate (Glu) is synthesized *de novo* from glucose (not shown) and from glutamine (Gln) supplied by glial cells. Glutamate is then packaged into synaptic vesicles by vesicular glutamate transporters (vGluTs). SNARE complex proteins mediate the interaction and fusion of vesicles with the presynaptic membrane. After release into the extracellular space, glutamate binds to ionotropic glutamate receptors (NMDA receptors (NMDARs) and AMPA receptors (AMPARs)) and metabotropic glutamate receptors (mGluR1 to mGluR8) on the membranes of both postsynaptic and presynaptic neurons and glial cells. Upon binding, the receptors initiate various responses, including membrane depolarization, activation of intracellular messenger cascades, modulation of local protein synthesis and, eventually, gene expression (not shown). Surface expression and function of NMDARs and AMPARs is dynamically regulated by protein synthesis and degradation and receptor trafficking between the postsynaptic membrane and endosomes. Glutamate is cleared from the synapse through excitatory amino acid transporters (EAATs) on neighbouring glial cells (EAAT1 and EAAT2) and, to a lesser extent, on neurons (EAAT3 and EAAT4). Within the glial cell, glutamate is converted to glutamine by glutamine synthetase and the glutamine is subsequently released by System N transporters and taken up by neurons through System A sodium-coupled amino acid transporters to complete the glutamate–glutamine cycle (from Popoli *et al.*, 2012)

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Glutamate is synthesized *de novo* from glucose in astrocytes via the Krebs cycle, followed by transamination or reductive amination of α-oxoglutarate, and it can be recycled through the glutamate-glutamine cycle (Erecinska and Silver, 1990). In glutamatergic synapses, presynaptic terminals are normally associated with specialized postsynaptic structures: the dendritic spines. The presynaptic machinery that modulates glutamate release through the vesicular transport is the so-called SNARE complex (Soluble N-ethylmaleimide-sensitive factor Attachment Protein REceptor). Two synaptic membrane proteins (syntaxin 1 or 2 and SNAP25) and a vesicular protein (synaptobrevin 1 or 2) interact to build up the SNARE complex; this complex mediates the fusion of synaptic vesicles with the presynaptic membranes (Lang and Jahn, 2008; Rizo and Rosenmund, 2008; Sudhof and Rothman, 2009). Glutamate regulates synaptic transmission and plasticity by activating ionotropic glutamate receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and NMDA) and metabotropic glutamate receptors (mGluR1 to mGluR8). The mGluRs are divided into three large groups including several sub-types of receptors distinguished by the specificity of theirs ligands (Nicoletti *et al.,* 2011) (Fig. 6). The number and stability of glutamate receptors at the synaptic membrane is an important factor in determining excitatory synaptic efficacy. Several mechanisms have been proposed to control the surface expression of NMDARs and AMPARs, including PDZ domain-mediated interactions between channel subunits and synaptic scaffolding proteins (Roche *et al.,* 2001; Hayashi *et al.,* 2000; Elias *et al.,* 2006), clathrin-dependent endocytosis regulated by phosphorylation (Lee *et al.,* 2002; Prybylowski *et al.,* 2005; Bhattacharyya *et al.,* 2009), and motor protein-based transport along microtubule or actin cytoskeletons (Setou *et al.,* 2002; Wang *et al.,* 2008). For example, combined β-adrenergic and corticosteroid receptor activation regulates AMPA receptor function in hippocampal neurons (Zhou *et al.* 2012). Members of the RAB family of small GTPases, which function as key regulators for all stages of membrane traffic, are involved in the internalization, recycling and delivery of NMDARs and AMPARs to the spine (Brown *et al.*, 2005; Park *et al.*, 2004). The synthesis and degradation of postsynaptic glutamate receptors are dynamically regulated (Liu *et al.*, 2010; Hawasli *et al.* 2007). Indeed, in order to avoid the phenomenon of excitotoxicicty (fig 5), the extracellular concentration of glutamate is regulated by high-affinity excitatory amino acid transporters (EAATs), which are located both on glial cells (EAAT1 and EAAT2) and on neurons (EAAT3 and EAAT4). This mechanism is necessary because of the lack of glutamate-specific degradative enzymes in the synapse (O'Shea, 2002).

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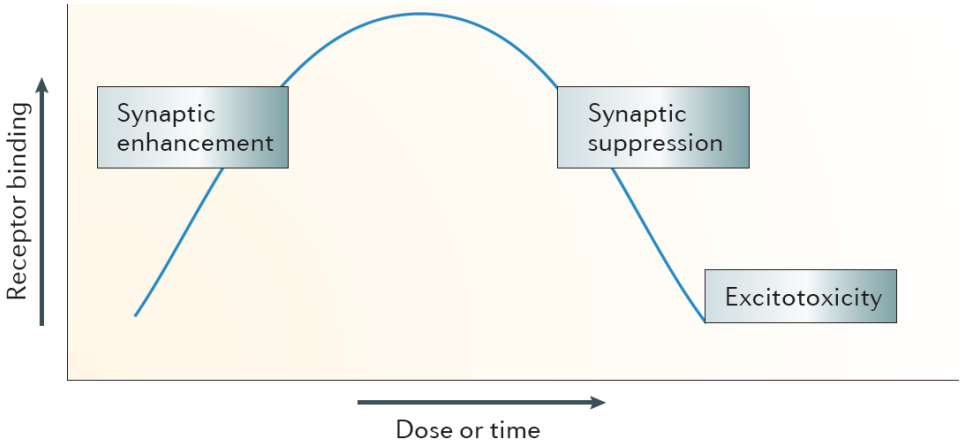

Figure 5: Glutamate-induced excitotoxicicty. Acute exposure to low or moderate physiological levels of adrenal steroids and EAAs (glutamate in this case) enhance synaptic function and certain types of memory, whereas higher levels of both mediators have the opposite effect. Conversely, chronic stress mediate adaptive plasticity involving spine synapse turnover, dendritic shrinkage and suppression of adult neurogenesis in the dentate gyrus. (Popoli *et al.*, 2012)

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Figure 6: Glutamate metabotropic receptors (adapted from Nicoletti *et al.*, 2011 ; Conn *et al.*, 2005; Swanson *et al.*, 2005).

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Glucocorticoids affect the basal release of glutamate in several brain regions, including hippocampus, amygdala and prefrontal cortex (Lowy *et al.*, 1993; 1995). Animal studies suggest that both acute and chronic stress may occur to modulate glucocorticoids response and, as a consequence, glutamate release. It has been shown that exposure of rats to acute stress such as, tail-pinch, forced-swim or restraint stress, induced a transient increase of extracellular glutamate levels in the prefrontal cortex (Moghaddam, 1993). In principle, the acute-stress-induced enhancement of stimulus-evoked release of glutamate may be achieved by increasing the number of synaptic vesicles that are already docked to the membrane and ready for release — the readily releasable pool (RRP) of vesicles — or by increasing the probability of release of synaptic vesicles, or both (Rizzoli *et al.*, 2005; Matz *et al.*, 2010; Lonart *et al.*, 2000). At the level of presynaptic machinery, footshock stress induced an increase in the number of SNARE complexes bound to the presynaptic membrane from prefrontal cortex neurons (Musazzi *et al.*, 2010) (Fig. 7), suggesting that at least the first mechanism is involved.

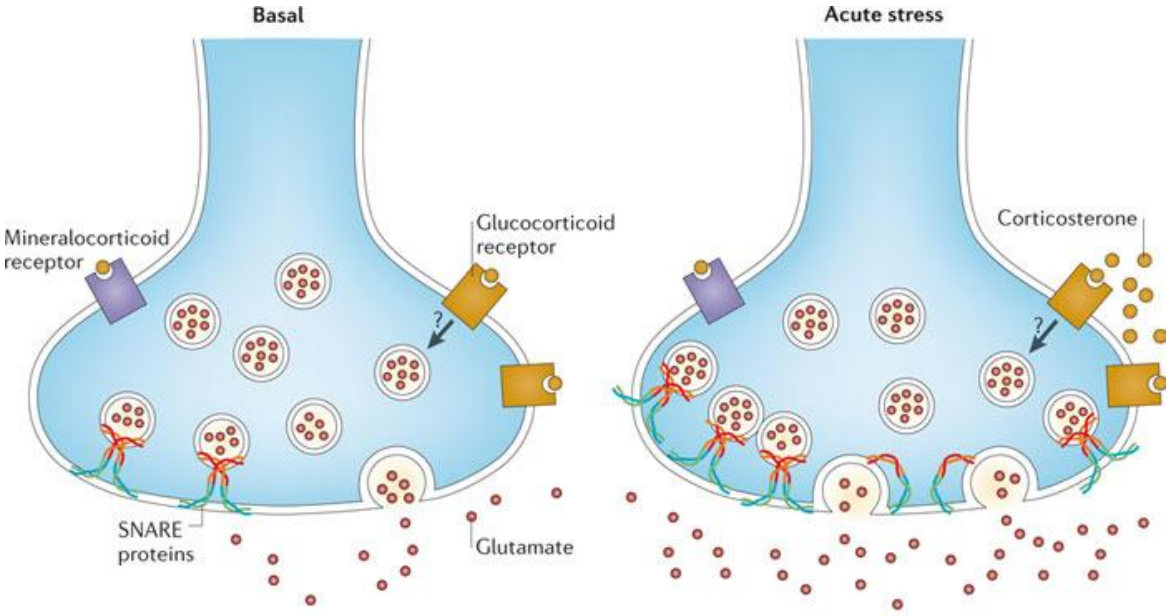

Figure 7: Glutamate release and acute stress. Acute stress both enhances depolarization-evoked release of glutamate from presynaptic terminals of rat prefrontal and frontal cortex and increases circulating levels of corticosterone. This induces a rapid glucocorticoid receptor-mediated increase of presynaptic SNARE protein complexes (which mediate fusion of synaptic vesicles) in the presynaptic membrane and an increase of the readily releasable pool of glutamate vesicles. (from Popoli *et al.*, 2012).

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In *in vitro* electrophysiological recordings, application of corticosterone to hippocampal slices rapidly increased the frequency of excitatory postsynaptic potential in CA1 pyramidal neurons (Karst *et al.*, 2005). It has been shown that three repeated tail-pinch stressors (at 2.5 hour intervals) in rats produced transient glutamate effluxes in the hippocampus (Moghaddam, 2002). One study showed a decrease in cortical glutamate uptake following 21 days of restraint-stress exposure (Olivenza *et al.*, 2000). A recent study also found a reduction in hippocampal glutamate clearance in hippocampal slice preparations from chronically stressed rats as well as evidence of increased glutamate release from hippocampal synaptosomes (de Vasconcellos-Bittencourt *et al.*, 2011). Emerging evidence suggests that glucocorticoids may have a role in mediating the effects of stress on EAAT2 regulation. Rats chronically exposed to high levels of glucocorticoids exhibited increases in the expression of GLT1b (an isoform of EAAT2 (which is also known as GLT1) in the hippocampus (Autry *et al.*, 2006). Yuen and coworkers (2009, 2011) have shown that exposing rodents to an acute stressor improves their performance in a working memory task, and this effect is abolished by blocking glucocorticoid receptor in the prefrontal cortex. This finding fits well with acute stress- or glucocorticoid-induced facilitation of working memory (which involves the prefrontal cortex) and declarative memory (which involves the hippocampus) observed in humans (Lupien *et al.*, 2002; Smeets *et al.*, 2006). By contrast, chronic stress or glucocorticoid treatment impairs prefrontal cortex-dependent cognitive functions in rats (Cerqueira *et al.*, 2005) and humans (Young *et al.*, 1999; Liston *et al.*, 2009), and likewise causes deficits in hippocampus-dependent cognitive processes (McEwen, 2007). However, the effects of chronic stress on glutamate are poorly known. There is growing evidence that chronic stress has effects on glial cell morphology, metabolism and function in the prefrontal cortex and possibly also the hippocampus. These long-lasting chronic stress-induced changes in glutamate transmission may be linked to the impairments in spatial and contextual memory performance and attentional control (McEwen 1999; Liston *et al.*, 2006) and the reduced synaptic plasticity in the hippocampus–prefrontal cortex connection that have been observed in rats after chronic stress (Cerqueira *et al.*, 2007). The decreased ability to clear extracellular glutamate as a result of impaired glial cell uptake and metabolism, combined with stress-induced changes in glutamate release and glutamate receptor function, could provide a pathophysiological mechanism leading to many of the structural changes observed in brain regions of individuals with stress-associated psychiatric disorders, such as mood and anxiety disorders.

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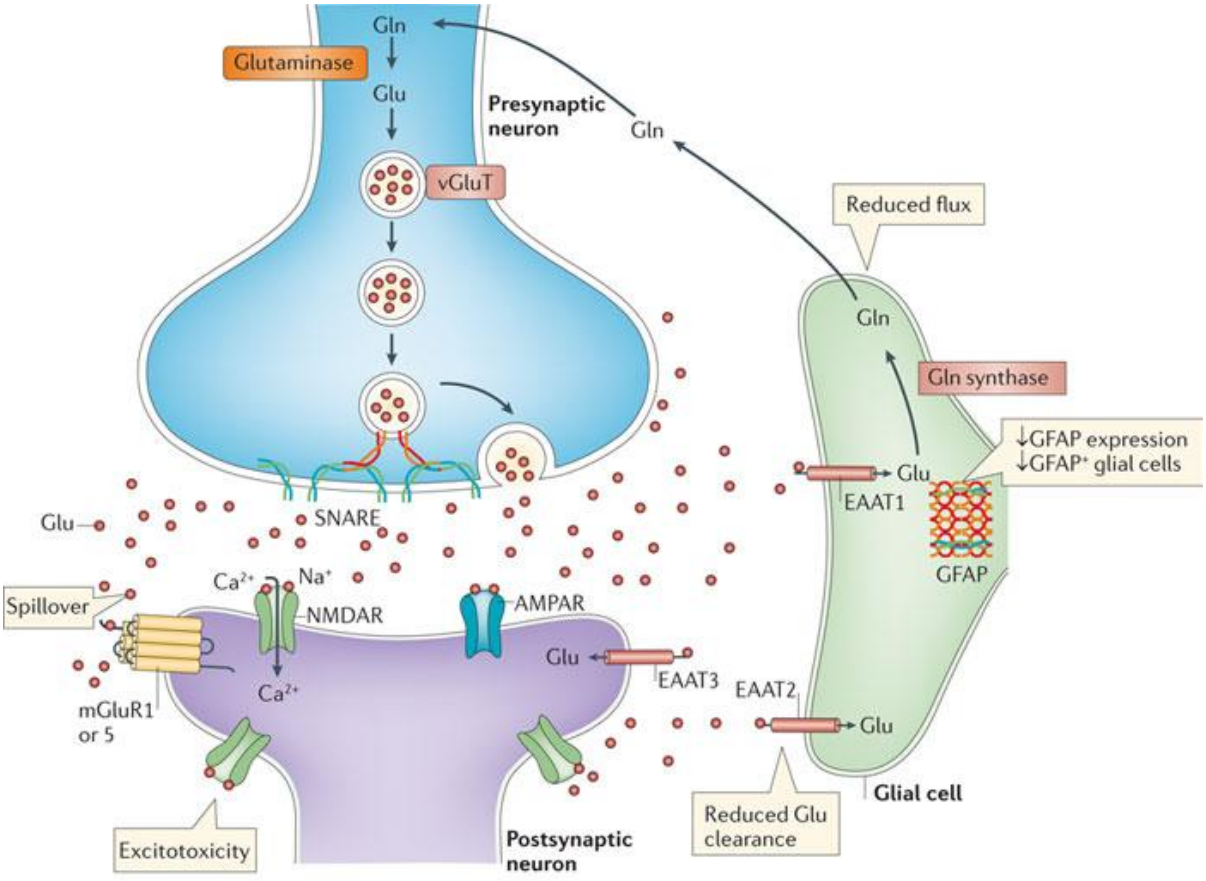

Figure 8: Chronic stress affects glutamate metabolism. Chronic stress may impair the ability to effectively clear synaptic glutamate (Glu) through glial EAATs. This may lead to glutamate spillover and, ultimately, increased activation of extrasynaptic glutamate receptors, resulting in excitotoxicity. Finally, chronic stress may decrease the rates of flux through the glutamate–glutamine (Gln) cycle, resulting in reduced glutamate metabolism. (from Popoli *et al.*, 2012)

1.2 Fetal and perinatal programming of adult diseases: clinical and preclinical data

The poet William Wordsworth wrote that "The Child is the father of the Man", and diseases may be no exception to this observation. Undoubtedly, stress is a major risk factor in the early programming of late life adaptations. In nature, a number of psychological stressors are anticipatory, such as unconditioned stimuli or species-specific behaviors, as for example, the avoiding of open spaces by rodents. Stressors can be acute or chronic and exposure to stressful events can touch all the life span, thereby affecting prenatal and postnatal life.

Table 2 shows the main physiological and anatomical effects of stress during different periods of life (Lupien *et al.*, 2009).

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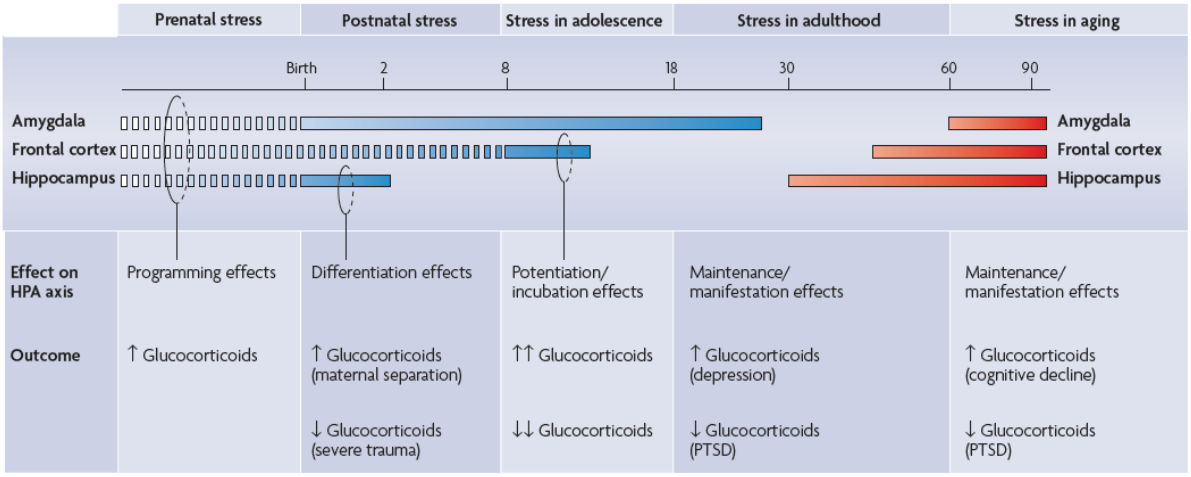

Table 2: The life cycle model of stress. How the effects of chronic or repeated exposure to stress (or a single exposure to severe stress) at different stages in life depend on the brain areas that are developing or declining at the time of the exposure. From the prenatal period onwards, all developing brain areas are sensitive to the effects of stress hormones (broken blue bars); however, some areas undergo rapid growth during a particular period (solid blue bars). In adulthood and during aging the brain regions that undergo the most rapid decline as a result of aging (red bars) are highly vulnerable to the effects of stress hormones. PTSD, post-traumatic stress disorder. (Lupien *et al.*, 2009)

The concept of fetal or developmental origins of health and disease was introduced to the medical community by epidemiological studies that linked the prevalence of different common adult disorders with body size at birth, a rough but convenient indicator of conditions during fetal life. For example, there are over 100 epidemiological studies in different populations (Barker *et al.*, 1989; 2005; Eriksson *et al.*, 2001) that have shown an association between small body size at birth in subjects born at term and increased risk of adult cardiovascular disease. The evidence is equally clear regarding type 2 diabetes, osteoporosis, schizophrenia and depression (Hales *et al.*, 1991; Newsome *et al.*, 2003; Dennison *et al.*, 2005; Wahlbeck *et al.*, 2001; Thompson *et al.*, 2001; Gale *et al.*, 2004).

The aforementioned epidemiological findings together with related experimental work have brought up the concept of programming—a process whereby a stimulus or insult, at a sensitive or 'critical' period of development, has lasting or lifelong significance. "Programming" may be on one hand advantageous in adjusting the metabolic needs or behavior of an individual for environmental conditions that are likely to prevail during the life-course. On the other hand, the effects may be harmful, for example, if the deficient nutritional conditions adjusted *in utero* are not sustained later in life. (Barker 1998; Gluckman and Hanson, 2004). Figure 9 summarizes current understanding on key mechanisms of programming.

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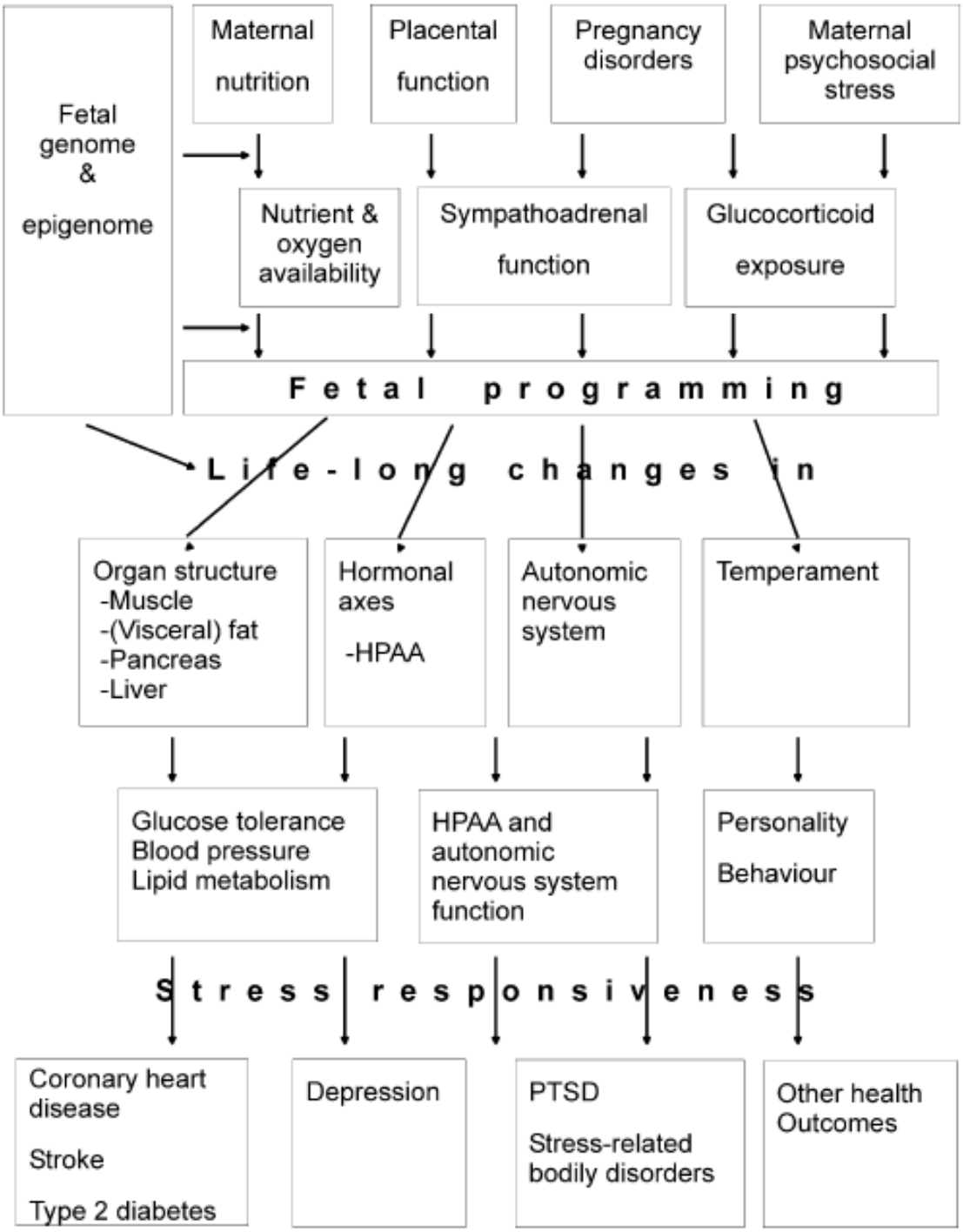

Figure 9. Conceptual model of possible pathways of fetal programming of stress-related adult disease. (from Kajantie, 2006)

Birth measurements or other retrospective data are only rough indicators of fetal environment and thus require large numbers of subjects to be studied. This limits the studies to phenotypes with data readily available from healthcare registers or obtained by simple clinical examinations or standardized questionnaires. However, for many stress-related disorders such as chronic fatigue, chronic pain, fibromyalgia posttraumatic stress disorder, data from

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healthcare registers are not available and diagnostic tests or surrogate assessments are more difficult to use in large-scale population studies. Consequently, there is little direct evidence linking these disorders with markers of intrauterine environment, such as size at birth. However, there is emerging evidence that the proposed endocrine mechanisms of stress-related bodily disorders, in particular hypocortisolism (Heim *et al.*, 2000) are related to conditions during fetal life (Kajantie *et al.*, 2002; 2003; Ward *et al.*, 2004). Moreover, it is widely acknowledged that events during early childhood have long-lasting effects on susceptibility to posttraumatic stress disorder, which is also characterized by hypocortisolism (Yehuda, 2002). Epidemiologic studies have evaluated the prevalence of stressors in the childhood family environment, including physical, sexual, and emotional abuse, neglect, exposure to family conflict and domestic violence, parental loss (separation, divorce, or death), parent psychopathology, and/or problematic parent– child relationships (Copeland *et al.*, 2009; Felitti *et al.*, 1998; Finkelhor *et al.*, 2005; Kessler *et al.*, 1997; Menard *et al.*, 2004). The percentage of adults reporting exposure to at least one childhood stressor has ranged from approximately half (Felitti *et al.*, 1998) to over three-quarters (Menard *et al.*, 2004) of community samples. Studies linking stress in the family context to children's negative psychological outcomes are numerous and span a range of developmental periods. Family stressors have been associated with internalizing and externalizing problems during early childhood (Shaw *et al.*, 1994), maladjustment during middle childhood (Rae-Grant *et al.*, 1989), and depressive symptoms during adolescence (Matjasko et al, 2007). A recent 45-year epidemiological study found that psychopathological consequences of childhood family adversity can persist through young adulthood and into middle age (Clark *et al.*, 2010). Notably, children of depressed parents may inherit genetic risk for emotion regulation deficits that typify depressive disorders (Goodman, 2007).

In nonhuman primates, emerging evidence suggests a critical period for some biological consequences within the first 6 months of life. Instead, for human children, a hyporesponsive period may occur throughout childhood, supported by parental regulation of the stress response (Lupien *et al.*, 2009). Thus, stressors related to inadequate parental care may elicit a stress response even during a stress hyporesponsive period. Animal models of early adversity often describe a "critical period" in development during which the stress must occur in order to impact stress physiology. For example, in rodents, noxious stimuli during the first 2 weeks of life have minimal effects on glucocorticoid response (Sapolsky and Meaney, 1986). Stress and glucocorticoids exposure during gestation results in low body weight at birth and elevated blood pressure, hyperglycemia, anxiety and increased HPA axis activity adulthood. Stress is tightly involved in the development and pathophysiology of the so called metabolic syndrome

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(Bjorntorp and Rosmond, 2000). Interestingly, depression is an important factor of comorbidity in metabolic syndromes (Brown *et al.*, 2004). This idea is supported by the finding that famine during the second or third trimester of fetal life is associated with increased risk of hospital treatment for major affective disorder (Brown *et al.*, 2000).

The development of epigenetic studies has significantly clarified the mechanism of early programming. Epigenetics signals refer to a series of chemical modifications to the DNA or to regions surrounding the DNA. Indeed, the classic epigenetic alteration is that of DNA methylation, which involves the addition of a methyl group (CH3) onto cytosines in the DNA. DNA methylation is associated with the silencing of gene transcription while histone acetylation at specific lysine sites is commonly associated with active gene transcription (Zhang and Meaney, 2010). Accordingly, recent observations showed nicely how lifelong effects could be set off by DNA methylation and maternal behavior in rats seems a key component in the investigation of these epigenetic programming effects. Indeed, pups subject to less licking and grooming were shown to have increased methylation of the glucocorticoid receptor gene promoter in the hippocampus, resulting in decreased transcription factor binding and consequently decreased HPA axis feedback inhibition. Interestingly, a causal role of DNA methylation was supported by the finding that the transcription factor binding was reversed by a histone deacetylase inhibitor, which in addition reversed the HPA axis stress response to the level of rats exposed to high licking grooming (Weaver *et al.*, 2004). Interestingly, maternal effects may modulate optimal cognitive functioning in environments varying in demand in later life, with offspring of high and low licking/grooming mothers showing enhanced learning under contexts of low and high stress, respectively (Champagne *et al.*, 2008)

1.3 Animal models of early stress

We have seen that several epidemiological studies on pregnant women have shown a high positive correlation between stressful events during the pregnancy and dramatic effects at birth, such as spontaneous abortion, preterm birth, low body weight, developmental delays and long-term behavioral abnormalities (Stott, 1973; Blomberg, 1980; Meijer, 1985; Homer *et al.*, 1990; Holmes, 2001; Weinstock, 2001). However, the investigation of the effects of early stress in humans results very difficult to perform, due to ethical concerns and retrospective approaches. Accordingly, during the last decades, scientists have developed specific animal models, especially in rats, where the stress is applied to the pregnant mother or early in life and the study is performed in the adult offspring. In rats, prenatal glucocorticoid exposure

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reduces birth weight in the new-born (Seckl, 2001), and small doses of the synthetic glucocorticoid dexamethasone prenatally given alter neuropsychological parameters such as emotionality (Trautman *et al.*, 1995; Lajic *et al.*, 1998). Also, prenatal exposure to dexamethasone reduces birth weight, affects brain development (Slotkin *et al.*, 1993) and programs hypertension and hyperglycaemia in adult offspring (Nyirenda and Seckl, 1998). Postnatal stress is also detrimental, in particular in early infancy which is a critical period during which the offspring almost entirely depends on parents or caregivers. Remarkably, while high level of active maternal behaviors such as licking-grooming and nursing has beneficial effects throughout life and in adulthood, low level can lead to depressive-like symptoms, anxiety, and altered synaptic plasticity and cognitive and social behaviors (Champagne *et al.*, 2008; Zhang *et al.*, 2010; Meaney, 2010; Bagot *et al.* 2009; Myers-Schulz and Koenigs, 2012). However the use of animal models of psychopathologies such as depression is risked with a singular anthropomorphic drift consisting in studying nonhuman-animal disorders defined by cognitive and emotional processes that are typically human (for example: depressed mood or self-esteem reduction in major depression). Nonetheless, by taking into account the necessary limitations, it remains possible to use animal models and, in particular, rodents for the investigation of cognition and behavioral disturbances. Indeed the following considerations are important to approach the research in animal models:

1. The assumptions on the psychological processes which cannot be measured in rodents must be eliminated from the field of investigation.

2. It is preferable to focus on the study of the symptoms instead of the syndrome. Rather than to observe the human syndrome overall and to compare it with the disorder presented by the model by making them correspond to a common etiology, one should focus on the analysis of each symptom and its origin.

3. A neurobehavioral mechanistic approach, in which neurobiological hypothesis rather than psychological hypothesis are posed as mechanisms for discrete symptoms, will yield more useful information concerning the nature and the treatment of depression (Holmes, 2003).

Here following a short presentation of the most studied animal models of perinatal stress, i.e. stress occurring during the prenatal and postnatal period.

Early handling

Early handling is a simple paradigm that consists in subjecting pups to short periods of separation from their mother during the first week(s) of life. This manipulation decreases overall stress responsiveness and favors a rapid surge and return to baseline of glucocorticoids immediately after stress (Levine, 1957; Cirulli *et al.*, 2003; Meaney *et al.*, 1996). Such fast adaptive response minimizes the risk of damage to the nervous system due to prolonged

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glucocorticoids exposure. It also reduces anxiety and enhances exploratory activity across life (Levine, 1957; Weinberg *et al.*, 1978). Early handling in rodents increases active maternal behaviors, which reduces HPA axis activity and can elicit stress resilience in the offspring when adult (Meaney *et al.*, 1996; Pryce *et al.*, 2001).

Maternal separation

Extended periods of maternal separation during postnatal life can persistently interfere with neurochemical, hormonal, and behavioral responses and induce stress vulnerability even in the absence of any direct manipulation of the pups (Moles *et al.*, 2004; Kikusui and Mori, 2009, Lucassen *et al.*, 2010). In rodents, 3 hours of daily separation from birth to 2 weeks postnatal can result in depressive-like behaviors upon re-exposure to stress later in life (Franklin *et al.*, 2011; Uchida *et al.*, 2010). At the behavioral level, male and female rats deprived from their mother at PND3 exhibit enhanced tone-cue fear conditioning responses (Oomen *et al.*, 2010, Oomen *et al.*, 2011 and Champagne *et al.*, 2008). These responses are mediated by the amygdala that is critically involved in fear and anxiety (LeDoux, 2007). Curiously, maternal deprivation do not affect the total branch length, number of branch points and primary dendrites or dendritic complexity index in the basolateral amygdala of male and female offspring (Krugers *et al.*, 2012). Maternal separation can have a strong or mild impact depending on its duration, frequency, and predictability. Nonetheless in some conditions, maternal separation can also be beneficial and promote stress resilience later in life. In rats, prolonged separation (6 hours) can lower emotional response and risk assessment and decrease anxiety in adverse conditions in adults (Roman *et al.*, 2006). Likewise, in mice, pups exposed to chronic unpredictable separation combined with maternal stress develop some resilience to social stress when adult (Franklin *et al.*, 2011).

Prenatal stress

Different procedures have been adopted to submit the pregnant rat to a stress condition (Weinstock, 2001, Baier *et al.*, 2012). The most frequent ones are the noise and flashing light stress applied on unpredictable basis three times a week throughout gestation (Fride and Weinstock, 1984), and prenatal restraint stress (PRS) applied three times a day during the last week or the last 11 days of gestation (Fig. 10) (Ward and Weisz, 1984; Maccari *et al.*, 1995; Alonso *et al.*, 1991). Interestingly, during this period, the fetal HPA axis begins to release its own ACTH and corticosterone (Boudouresque *et al.*, 1988). PRS paradigm constitutes the stress procedure that has been adopted in all of the studies described in the present thesis.

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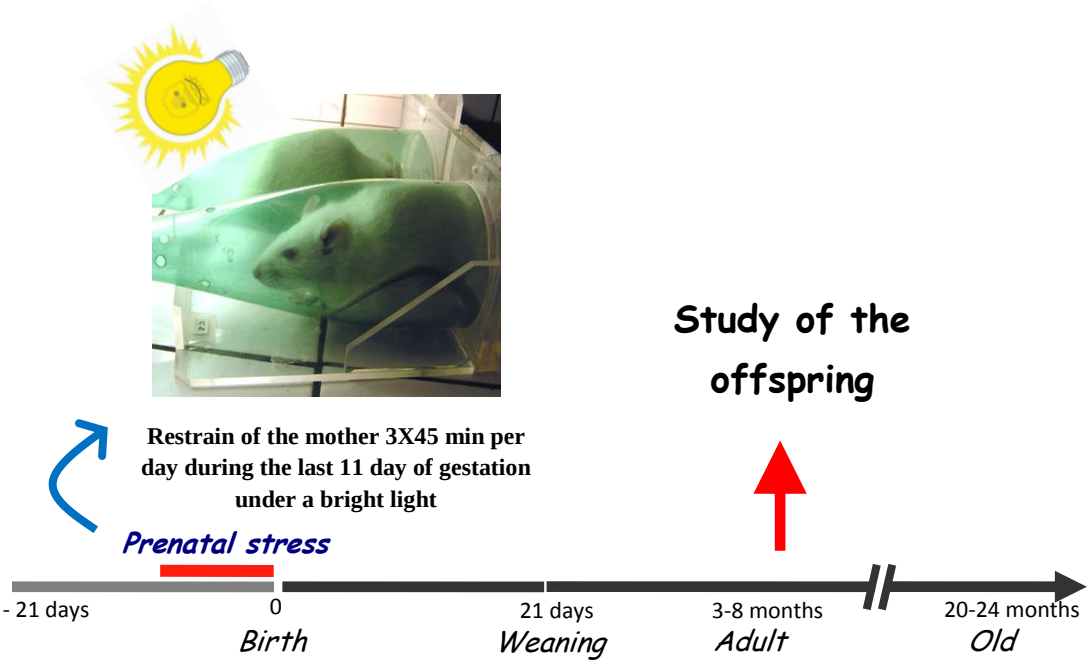

Figure 10: The prenatal restraint stress procedure. Animals are subjected to PRS according to our standard protocol (Maccari *et al.*, 1995; Morley-Fletcher *et al.*, 2003). From day 11 of pregnancy until delivery, pregnant female rats undergo to three stress sessions daily (45 min. each), during which they are placed in transparent plastic cylinders and exposed to bright light. Only male offspring from litters containing 10-14 pups with a comparable number of males and females are used for the experiments. A maximum of one or two male pups are taken from each litter for each measure to remove any litter effects (Becker and Kowall, 1977; Chapman and Stern, 1979).

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2 Prenatal restraint stress and early programming of mood disorders

2.1 PRS affects HPA axis and neurochemical homeostasis

2.1.1 PRS and HPA axis

In rat offspring, there is evidence of an abnormal regulation of the HPA axis induced by maternal stress. PRS increases stress-induced corticosterone secretion in pre-weaning rats (Henry, 1994) and induces a prolonged post-stress corticosterone secretion in adult animals (Fride *et al.* 1986). A number of studies have also shown that adult PRS rats present decreased binding ability of hippocampal MR and GR when compared to control animals (Weinstock *et al.*, 1992; Henry *et al.*, 1994; Maccari *et al.*, 1995; Barbazanges *et al.* 1996b; Koehl *et al.*, 1997). Although these data provide evidence for an impairment of negative feedback control of HPA axis in PRS rats, they suggest that the change in corticosterone receptors is the result of the elevation of circulating GCs and not its cause (Maccari *et al.*, 1995) Accodingly, Maccari and coworkers (1995) found that (i) prenatal stress prolongs stress-induced corticosterone secretion in adult rats, which was attributed to the observed decrease in central corticosteroid receptors; (ii) adoption, irrespective of the stress experience of the foster mother, reverses the effects of prenatal stress; and (iii) adoption per se increases maternal behavior and decreases the stress-induced corticosterone secretion peak in the adult offspring (Fig. 11 A,B). Also, PRS enhances the increase in circulating glucocorticoid levels associated with ageing (Sapolsky, 1992), as middle-age PRS rats exhibited an increased basal secretion of corticosterone similar to levels found in old control animals (Vallée *et al.*, 1999). This could occur through a failure of the normal adaptation process as described above, and it is supported by the observation that 3 days-old PRS pups were found to have higher plasma corticosterone than control pups after stress, despite the presence of a similar number of hippocampal corticosteroid receptors (Henry *et al.*, 1994) (Fig.12).

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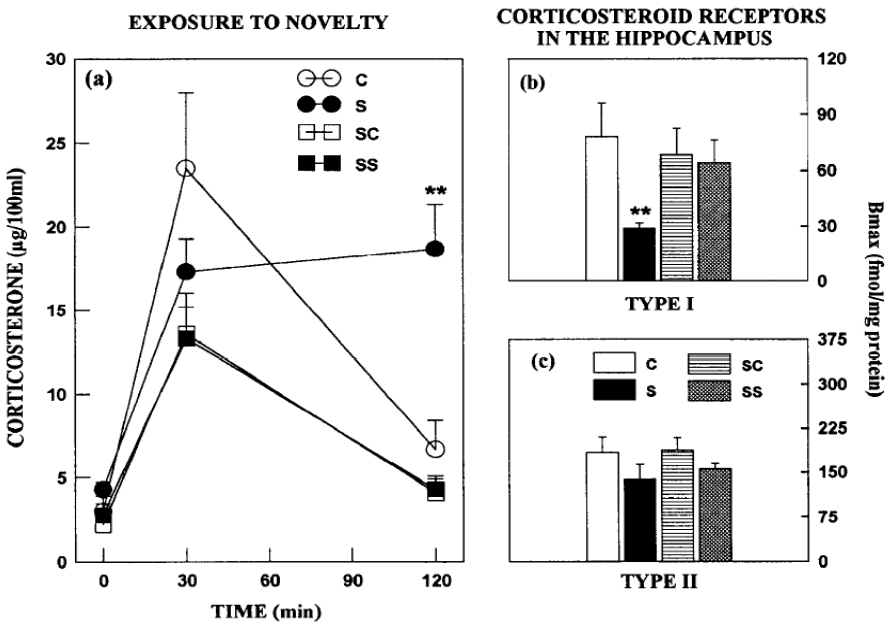

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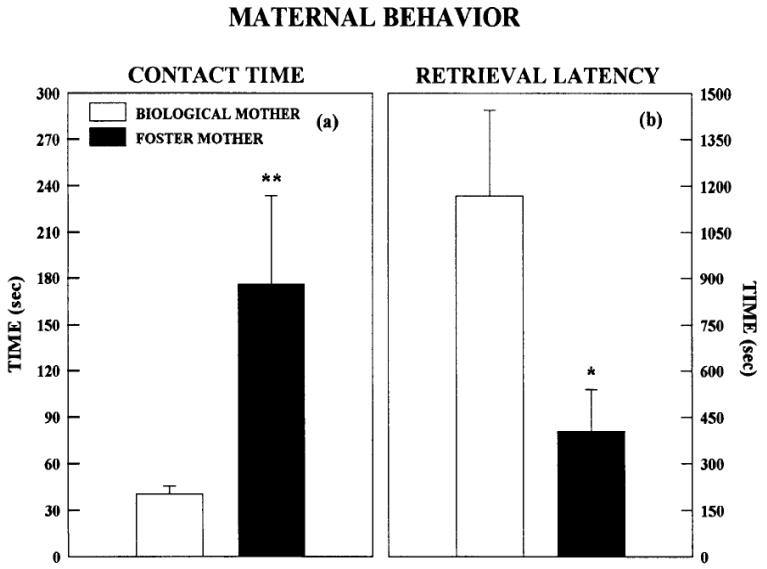

Figure 11: **(A)** Plasma corticosterone secretion after novelty exposure (a) and type I (b) and type II (c) corticosteroid receptors in adult prenatally unstressed rats raised by their biological mother (C), adult prenatally stressed rat raised by their biological mother (S), adult prenatally stressed rats adopted by a control unstressed mother (SC), and adult prenatally stressed rats adopted by a mother stressed during pregnancy (SS). a, Prenatally stressed animals (S) displayed higher corticosterone levels than those of control rats (C) after 120 min of novelty exposure. Animals that were both stressed and adopted did not differ from controls, either ifthe adoptive mother was unstressed (SC) or stressed (SS) during pregnancy. b, Type I corticosteroid receptors were reduced by prenatal stress and this effect was totally reversed by adoption in both SC and SS groups. c, Neither prenatal stress nor adoption significantly modified type II corticosterone receptors. **(B)** Effects of adoption on maternal behavior. a, Foster mothers spent longer licking and picking up the pups than did biological mothers. b, Latency to replace all the pups in the nest was lower in foster than in adopted mothers (from Maccari *et al.*, 1995).

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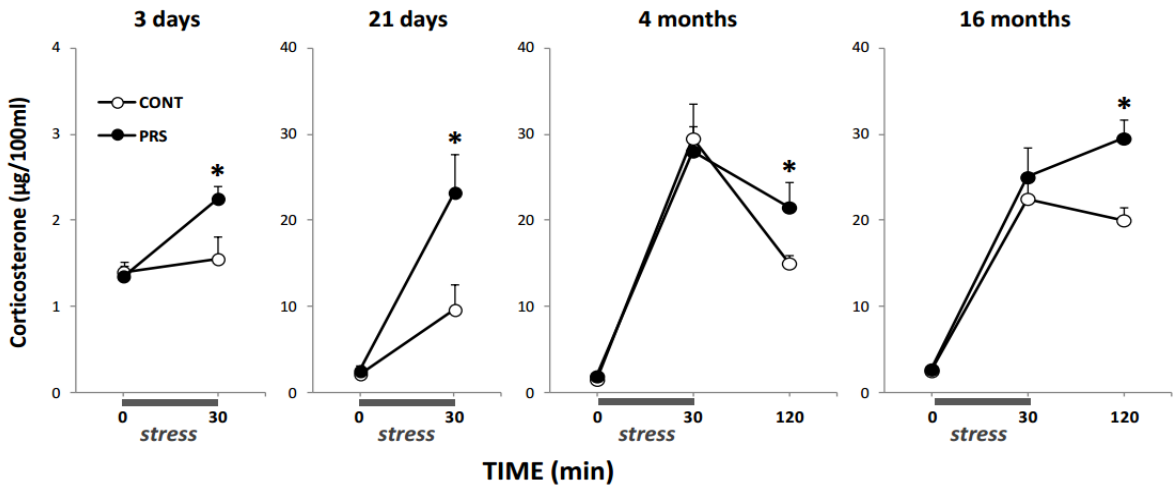

Figure 12: PRS enhances corticosterone response to stress during life span (adapted from Henry *et al.*, 1994; Vallée *et al.*, 1997).

2.1.2 Neurochemistry

In rats, PRS has been reported to affect the serotonin (5-HT) system, with increased or decreased levels of 5-HT contents in the cortex and in the hippocampus respectively (Peters, 1986; 1988; 1990). The change in 5-HT function could be involved in the alterations observed at the HPA axis levels; indeed, there is a reciprocal influence between these two systems (Joëls *et al.*, 1991; de Kloet *et al.*, 1998). PRS rats showed an increased expression of the 5HT-1A receptors in the cortex (Morley-Fletcher *et al.*, 2004a) and the hippocampus (Hayashi *et al.*, 1998). In addition, PRS rats are characterized by an increase number of the post-synaptic 5HT-2 receptors (Peters, 1986; 1988; 1990). Another very recent study shows a decreased level of 5-HT1A immunobinding in the ventral hippocampus, which is primarily implicated in emotional processing, and this decrease was more important for male than for female PRS rats (Van den Hove *et al.*, 2006). Moreover, long-term effects on the development of the forebrain cholinergic system have been observed (Day *et al.*, 1998) with increased hippocampal acetylcholine release following stress or injection with corticotropin-releasing factor (CRF) (Fig. 13) This hormone has been found to be increased in the amygdala of PRS rats (Cratty *et al.*, 1995). Reduced contents and lower turnover of noradrenalin and dopamine have been reported (Fride and Weinstock, 1988; Takahashi *et al.*, 1992; Henry *et al.*, 1995). In this regard, it has been found that in male rats PRS eliminated the asymmetries in striatal dopaminergic function and reduced the asymmetries in the size of cerebral cortex (Alonso *et al.*, 1991b; 1994; 1997; Fleming *et al.*, 1986). Like human subjects, normal rats display cerebral asymmetries which are related to patterns of brain organization and control of various behavioral functions (Carlson and Glick, 1989). These specific alterations induced by gestational stress have been proposed to parallel the clinical observations of an interference

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with the reduction of cerebral asymmetries observed schizophrenic patients (Weinstock, 2001). The changes observed at the levels of the dopaminergic system have also important implications in the development of an increased sensitivity to psychostimulants reported in these animals (Deminiere *et al.*, 1992; Henry *et al.*, 1995; Koehl et al, 2000).

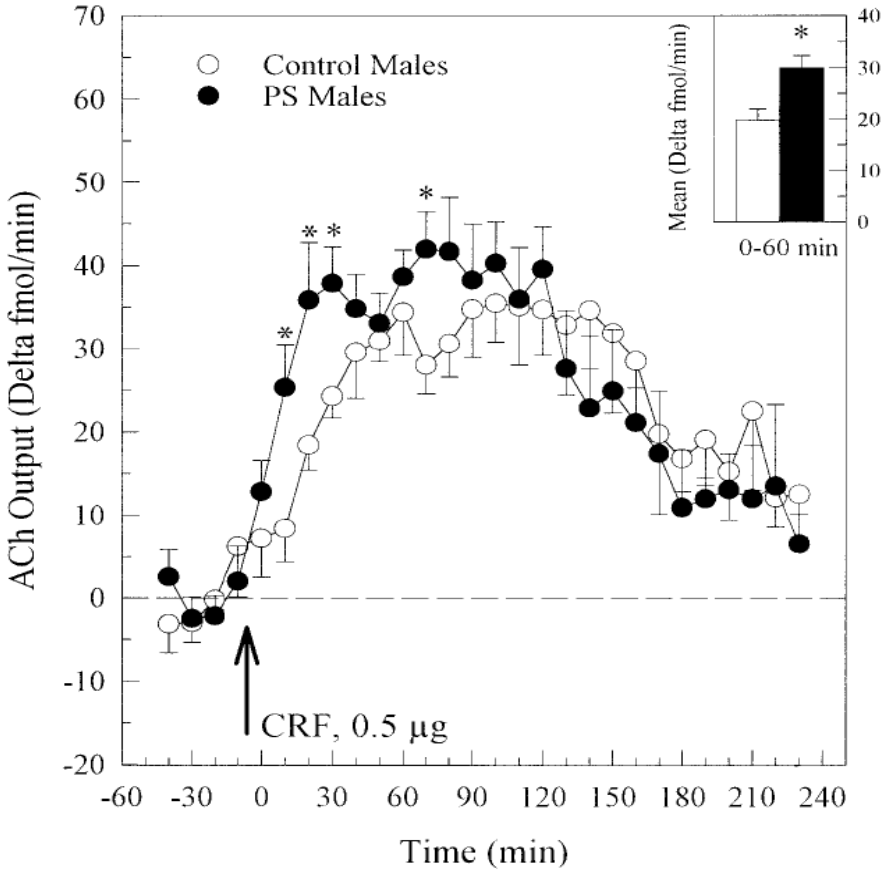

Figure 13: Effects of prenatal stress on CRF-induced hippocampal ACh output (Δ fmol/min) in males. Prenatally stressed rats showed a higher ACh response to CRF than control rats over the first hour (inset; *p , 0.05). This difference was attributable to a higher ACh release 10, 20, 30, and 70 min after CRF injection. (from Day *et al.*, 1998).

The noradrenergic neurons of the locus coeruleus (LC) constitute the central component of the sympathetic nervous system involve in the stress response as in the attention processes (Morilak *et al.*, 2005). PRS increases the hypothalamic basal NA concentrations (Peters, 1982) in CNS whereas, at plasmatic level, PRS rats show the same basal NA levels than control rats (Weinstock *et al.*, 1998). Interestingly, after an electric shock, PRS rats present an increased concentration of NA and its metabolites (Weinstock *et al.*, 1998). This suggests a hyperactivity of the NA system in PRS rats. Recently, it has been shown that PRS modifies prefrontal cortex catecholamine transmission in a complex and age dependent manner (Carboni *et al.*, 2010) and that it may produce a complex change in accumbal dopamine and

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noradrenaline transmission (Silvagni *et al.*, 2008). Interestingly, the expression of both Nurr1 and Pitx3, two transcription factors that are expressed at critical periods of DA neuron differentiation, are increased in the ventral tegmental area of PRS rats (Katunar *et al.*, 2009).

Few works have explored the glutamatergic and GABAergic systems in PRS rats. We have recently shown that PRS exhibit a reduced expression of the γ2 subunit of $GABA_A$ receptors in the amygdala at PND14 and PND22, an increased expression of mGlu5 receptors in the amygdala at PND22, a reduced expression of mGlu5 receptors in the hippocampus at PND14 and PND22, and a reduced expression of mGlu2/3 receptors in the hippocampus at PND22 (Laloux *et al.*, 2012). Although glutamate is one of the most represented neurotransmitter in the brain (particularly in hippocampus), the high diversity of function of glutamatergic neurotransmission (especially under stress condition) is still at its infancy. It has been shown that PRS rats presented an increase of NMDA receptors expression in the cortex, hippocampus and in various striatal areas (Barros *et al.*, 2004). Mounting evidence suggests that acute and chronic stress, especially the stress-induced release of glucocorticoids, induces changes in glutamate neurotransmission in the prefrontal cortex and the hippocampus, thereby influencing some aspects of cognitive processing. In addition, dysfunction of glutamatergic neurotransmission is increasingly considered to be a core feature of stress-related mental illnesses. Recent studies have shed light on the mechanisms by which stress and glucocorticoids affect glutamate transmission, including effects on glutamate release, glutamate receptors and glutamate clearance and metabolism (Popoli *et al.*, 2012). Interestingly, our group has uncovered a pronounced gender difference in the effects of PRS on mGlu receptors and a series of neurobiological parameters classically associated with hippocampus-dependent behaviors. Adult male rats subjected to PRS showed a reduction in the survival of newborn cells in the dentate gyrus, a reduction in the activity of mGlu1/5 receptors in the ventral hippocampus, and an increase in the levels of brain-derived neurotrophic factor (BDNF) and pro-BDNF in the hippocampus. In contrast, female PRS rats displayed improved learning in the Morris water maze, an increase in the activity of mGlu1/5 receptors in the ventral and dorsal hippocampus, and no changes in hippocampal neurogenesis or BDNF levels (Zuena *et al.*, 2008) (Fig. 14). Recently, we have shown that ethanol intake resulted in differential effects in the expression of mGlu receptor subtypes implicated in mechanisms of learning and memory. In control rats, ethanol intake reduced mGlu2/3 and mGlu5 receptor levels in the hippocampus; in PRS rats, which exhibited a constitutive reduction in the levels of these mGlu receptor subtypes, ethanol increased the expression of mGlu1a receptors but did not change the expression of mGlu2/3 or mGlu5 receptors (Van Waes *et al.*, 2011).

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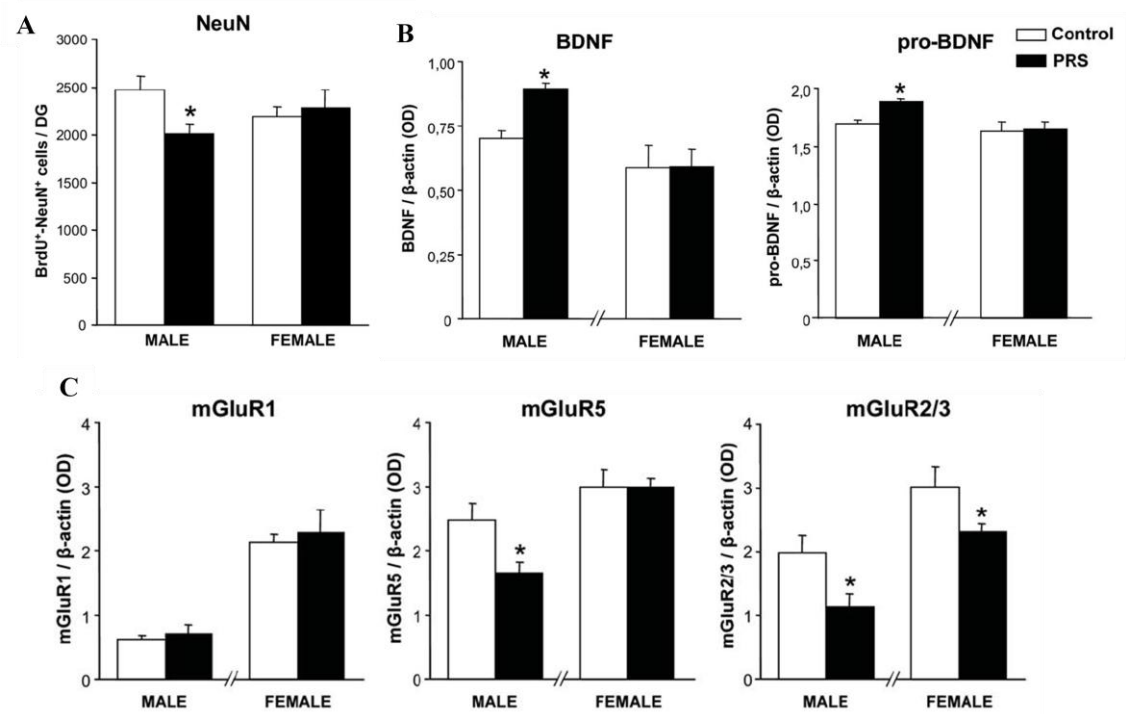

Figure 14: (A) Hippocampal neurogenesis and gliogenesis in control and PRS rats. NeuN (neuronal marker) DG: dentate gyrus; PRS reduced the total number of BrdU+/NeuN+ cells in male rats (* $p<0.05$), with no effect in female rats. **(B) PRS enhances hippocampal BDNF levels in male rats.** PRS increased the steady-state levels of both BDNF and pro-BDNF in male rats (* $p<0.05$) but had no effect in female rats. **(C) Expression of group-I and group–II mGlu receptors in the hippocampus of control and PRS rats.** In male rats, PRS induced a reduction in the expression of mGlu5 receptors (* $p<0.05$) but no change in the expression of mGlu1a receptors. PRS had no effect on the expression of mGlu1a and mGlu5 receptors in female rats. PRS reduced mGlu 2/3 receptor expression in the hippocampus of both male and female rats (* $p<0.05$). (adapted from Zuena *et al.*, 2008).

2.2 PRS as a model to study depression and mood disorders

2.2.1 The anxious/depressive-like behaviors

Exposure to prenatal stress induces long-lasting effects on the behavior, causing a general impairment of the adaptive capabilities of the individual (Weinstock, 2001).

In animal studies, stress during pregnancy reduces testosterone surge at birth in male rats (Ward, 1972), with a consequent impairment of sexual activity (Masterpasqua *et al.*, 1976; Ward, 1983). Alterations in the early motor development have also been reported (Barlow, 1978). PRS reduces in males the amounts of play behavior to levels expressed by females (Ward and Stehm, 1991), thus eliminating the sex differences that are normally observed for this behavioral pattern (Meaney, 1989). Prenatally stressed monkeys display a reduced play

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behavior and exploration of their surroundings than control animals and this has been shown to be combined to an increase in clinging to other monkeys, a sign of greater anxiety in the face of novelty. This finding has been also confirmed in humans by Meijer (1985) which showed that prenatally stressed children (children who were in their first half year of life at the time of the war) were less sociable than their peers.

In control animals, the response to fear-provoking situations shows an inverted U-shaped function between activity and fear. However, adult PRS rats (Ward and Weisz, 1984; Wakshlask and Weinstock, 1990; Poltyrev *et al.*, 1996; Vallée *et al.*, 1997) and monkeys (Schneider, 1992), show less exploration and more defecation and escape behaviour than controls in an intimidating novel environment. Such differences can be abolished by a treatment with anxiolytic drugs like benzodiazepines (Pohorecky and Roberts, 1991; Drago *et al.*, 1999). Moreover, Vallée and coworkers (1997) have demonstrated that the effects of PRS on several features of emotional behavior were strongly correlated with post-stress levels of corticosterone. In conclusion, the alterations of the HPA axis activity induced by PRS can be associated to the changes observed in adult behavioral reactivity.

The reduced consumption of appetitive substances like sweetened water is used to model and to evaluate the anhedonia-like symptoms, one of the main feature of depressive-like disorders (Katz 1982). Indeed, reduced sucrose consumption was observed in rats subjected to chronic stress and this reduced consumption can be restored by various chronic antidepressant treatments (Willner *et al.*, 1987; Moreau *et al.*, 1994). PRS decreases the sucrose consumption in the females and increases it in males (Keshet *et al.*, 1995). Several studies highlighted that the PRS exacerbates the phenomenon of learned resignation in response to uncontrollable electric shocks (Secoli *et al.*, 1998) and increases time spend in immobility in the Porsolt test (Alonso *et al.*, 1991; Drago *et al.*, 1999; Morley-Fletcher *et al.*, 2003; 2004a).

PRS increased the time spend in immobility in response to an electric shock (Takahashi *et al.*, 1992), indicating that PRS rats have a higher propensity to behavioral inhibition in this situation of intense stress. PRS increased fear and anxiety by exhibiting a reduction of exploration and an increase of the defecation in the open field test (Archer *et al.*, 1971; Vallée *et al.*, 1997; Weinstock *et al.*, 1992). However other studies show a behavioral hyperactivity in particular during the first minutes of various behavioral tests as the Y maze (Deminiere *et al.*, 1992). The differences of experimental conditions and the variety behavioural tests used could contribute to this *gamma* of results. In the elevated plus maze, PRS rats spend less time in the open arms, indicating an increased anxiety-like behaviours (Poltyrev *et al.*, 1996; Vallée *et al.*, 1997; Wakshlak *et al.*, 1990; Rimondini *et al.*, 2003; Zimmerberg *et al.*, 1998). In the test of the light and dark box, PRS induces a reduction in the number of entry and time

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spent in the white compartment (Ward *et al.*, 2000). The increased anxiety-like behavior is also detectable by a reduced social interaction in PRS young rats (Ward *et al.*, 1991; Morley-Fletcher *et al.*, 2003) and in adult female rats (Weinstock, 2001).

2.2.2 Cognitive disorders

PRS affects the spatial learning in the adult male rats in the swimming test (Morris test) a hippocampal-dependent test of spatial learning (Lemaire *et al.*, 2000) and the water-filled T-maze (Nishio *et al.*, 2001). Conversely, PRS does not affect neither the acquisition of an operant conditioning nor the associated discrimination task, but it delays the learning of a new discrimination task (Weller *et al.*, 1988). In addition, PRS influences the contextual fear conditioning by increasing the conditioned response of immobility (Shalev and Weiner, 2001). However, PRS delays the learning of passive (Drago *et al.*, 1999) and active avoidance tasks (Lehmann *et al.*, 2000). These alterations are in accordance with attention deficits. Latent inhibition paradigm can be used to assess the attention performance (Solomon *et al.*, 1980). Although several studies show that latent inhibition was not affected by PRS (Shalev and Weiner, 2001), other work suggest a sex-specific effect with an increased latent inhibition in males PRS rat (Bethus *et al.*, 2005). According to Lemaire and coworkers, the mnemonic effects induced by the PRS are highlighted by a decreased hippocampal neurogenesis during learning (Lemaire *et al.*, 2000). In addition, ageing implies at the same time a reduction of the hippocampal neurogenesis and a decrease of the mnemonic performances. Accordingly, Vallée and coworkers showed that the PRS potentiated in male rats the mnemonic age-related deficits related in the test of spontaneous space recognition (the Y maze), but have no effect on the Morris water maze test performances (Vallée *et al.*, 1999). On the other hand, Darnaudéry and coworkers (2006) showed that a PRS increases the mnemonic deficits related in 24 months old female in the Morris water maze test.

2.2.3 Impairment of circadian rhythms

Many of the physiological or behavioral processes within the organism fluctuate dramatically on a regular basis throughout the 24-hours day. This daily rhythm arises from an internal time-keeping system, the circadian clock, located in the hypothalamic suprachiasmatic nuclei (Stephan and Zucker, 1972; Turek and Van Reeth, 1995). In the absence of environmental inputs, these rhythms persist with a period of about 24 hours and are therefore referred as circadian rhythms. In addition to changes in the light–dark cycle (Pittendrigh, 1981), circadian function and sleep patterns are also regulated by neurochemical or behavioral stimuli (Van Reeth and Turek, 1989). Among these stimuli, steroids have a marked effect on the functioning of the circadian system (Turek and Gwinner, 1982) and chronic stress in adult rats can induce changes in circadian rhythms as well as in sleep patterns (Kant *et al.*, 1995;

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Cespuglio *et al.*, 1995). Accordingly, it has been shown that PRS in rats can induce an advanced shift in corticosterone secretion, with higher levels of total and free corticosterone secreted at the end of the light period in both sexes and increased corticosterone secretion over the entire diurnal cycle in females (Koehl *et al.*, 1997; 1999). The effects of PRS on the rhythm of corticosterone secretion could be mediated by a reduction in hippocampal corticosteroid receptor expression at specific times of the day. Indeed, reduced MRs at the beginning of the light phase has been observed in males and at the end of the light phase in both sexes (Koehl *et al.*, 1999). Disturbances in the circadian rhythm of locomotor activity have also been reported in PRS rats. These include a reduced rate of resynchronisation of the activity rhythm after an abrupt shift in the light dark cycle (Van Reeth *et al.*, 1998) and a phase advance in the rhythm of wheel running behaviour (Koehl *et al.*, 1999).

Another important alteration induced by PRS on circadian timing includes changes in sleep-wake parameters that are observed in adult animals (Dugovic, *et al.* 1999. Under baseline conditions PRS rats showed increased amounts of paradoxical sleep, positively correlated to plasma corticosterone levels. Other modifications include increased sleep fragmentation, increased total light slow-wave sleep time and a slight decrease in the percentage of deep slow-wave relative to total sleep time) (Fig. 15). During recovery sleep following acute restraint stress, all sleep changes persisted and were correlated with stress-induced corticosterone secretion. High corticosterone levels under baseline conditions as well as under acute stress may thus predict long-term sleep-wake alterations. In addition to GCs, other factors may be involved in the long-term effects of PRS on sleep. The serotonin system is another good candidate, as exposure to high glucocorticoid levels or acute stressors results in significant alterations in 5-HT turnover in the midbrain/pons area of PRS rats (Muneoka *et al.*, 1997). As reported above, PRS induces long-term altered response to 5-HT receptor agonists (Peters, 1988). Concerning this role played by the 5-HT system on the regulation of paradoxical sleep and on sleep-wake modulation (Jouvet, 1969; Boutrel *et al.*, 1999; 2002), developmental alterations in brain 5-HT metabolism may contribute to the modification in sleep parameters induced by PRS.

Abnormalities in circadian rhythm have been described in the majority of depressed patients (Rosenwasser and Wirz-Justice, 1997). As a consequence, an altered pattern of circadian rhythms combined with an overall impairment of the HPA axis and behavioral and neurochemical alterations suggest that, nowadays, PRS is a well characterized model of depression.

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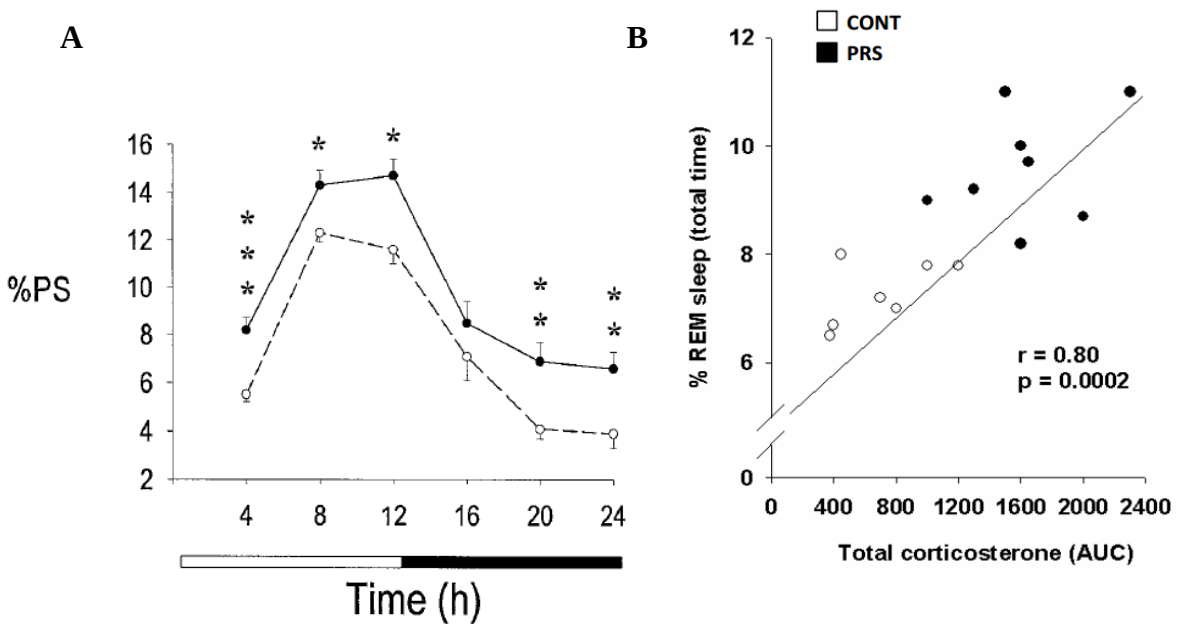

Figure 15: (A) Distribution per 4 hr intervals of vigilance states in control (white circles) and PRS (black circles) rats under baseline conditions. Paradoxical sleep (PS) is expressed as percentage of recording time. *p , 0.05; **p , 0.01; ***p , 0.001. **(B) Positive correlations between individual stress-induced plasma corticosterone AUC values and amounts of PS.** (adapted from Dugovic *et al.*, 1999)

2.2.4 Pharmacological validity of the PRS model by antidepressants

The persistence of the abnormalities induced by PRS could be an interesting tool to test novel pharmacological strategies concerning mood and depressive-like disorders. Indeed, the symptoms presented by PRS rats are persistent during all life span and thus facilitate the study of the effects of chronic pharmacological treatments for depression.

Willner (1997) proposed three criteria to validate an animal model of depression: face, predictive and construct validity. Face validity refers to the phenomenological similarity to humans whereas predictive validity refers to the accuracy of a model in forecasting the course and outcome of a human syndrome. Finally, construct validity represents the degree to which both the human syndrome and the animal model are unambiguously defined such that a rational theory can be constructed to explain the pathophysiology of disorder. This last criterion is founded on the knowledge of the causes of the psychiatric illnesses. However, because mental disorders seem human-related pathologies, the perfect homology of an animal model to a human psychiatric condition cannot be absolutely demonstrated. In contrast, it is possible to use animal models to highlight symptoms and develop new pharmacological strategies.

- Face validity. As reported above, several studies have established that PRS rats present an impaired feedback inhibition of HPA axis activity (Henry *et al.*, 1994; Maccari *et al.*, 1995; Barbazanges *et al.*, 1996; Koehl *et al.*, 1997; 1999) and increased levels of CRF in amygdala

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(Cratty *et al.*, 1995). Moreover, in accordance with the observed dysfunctions in the serotonergic system observed for several depressed patients (Meltzer and Lowy, 1987), PRS rats exhibit increased levels of postsynaptic 5-HT2 receptors (Peters, 1986; 1988; 1990).

Consistently with the alteration in sleep-wake cycle regulation and increases in paradoxical sleep reported in depressed humans (Poland *et al.*, 1992), PRS adult rats present persistent changes in sleep architecture that parallel those found in depressed patients (Dugovic *et al.*, 1999). Significant correlations between sleep abnormalities and dysfunction of the HPA axis have been found in depressed patients (Poland *et al.*, 1992; Hubain *et al.*, 1998) and may result from a stress component (Rosenwasser and Wirz-Justice, 1997). Interestingly, the persistence of paradoxical sleep alterations observed in the PRS model are dramatically different from the temporary sleep abnormalities observed in other stress models such as the chronic mild stress model (Cheeta *et al.*, 1997; Moreau *et al.*, 1995), in which paradoxical sleep is increased only during the first day of stress recovery and disappears soon after stress termination. It has been shown that PRS, in rat, induces lifespan reduction of neurogenesis in the hippocampus (Lemaire *et al.*, 2000). This is consistent with stress-induced structural remodeling in the hippocampus that can characterize the impairment of neural plasticity in the brain of depressed patients (Sheline *et al.*, 1996; Sapolski, 2000; McEwen, 2001). From a behavioral point of view, PRS rats exhibit anxiety-like behavior (Vallée *et al.*, 1997; Weinstock *et al.*, 2001) and comorbidity with anxiety is often associated to human depression (Stahl, 1993; Rouillon, 1999). Furthermore, Alonso and coworkers (1991; 1997) have shown that PRS female rats exhibit behavioral despair in the forced swim test, a test classically used to validate the efficacy of antidepressants (Porsolt, 1978). Taken together, these results reinforce the idea that the PRS model in the rat could be a suitable animal model of depression. Importantly, in this model, the alterations reported are stable throughout life-span, since they can be observed at early (Henry *et al.*, 1994) as well as later stages of development (Vallée *et al.*, 1999). These data suggest that the PRS model meets requirements of face validity.

- Predictive validity. Several work completed in our laboratory, thus, attempted to determine the effects of chronic antidepressants treatment on PRS rats. Indeed, imipramine (tryciclic), tianeptine (a selective serotonin reuptake enhancer, structurally similar to the tricyclic antidepressants) or agomelatine (an antidepressant with melatonergic agonist and 5-HT2C antagonist properties, see below) reverse several PRS-induced alterations at the behavioral, neurochemical and neuroanatomical level. Thus, following antidepressant treatment, PRS rats displayed reduced immobility behaviour in the forced swim test, increased exploration of the open arm in the elevated plus maze, enhanced mineralocorticoid and glucocorticoid receptors

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densities in the hippocampus and, modified 5-HT1A mRNA expression (Morley-Fletcher *et al.*, 2003, 2004a). Also, since preclinical and clinical research has increasingly focused on the interaction between stress and depression and their effect on hippocampus (Duman *et al.*, 2001), the effects of antidepressant treatment on hippocampal neurogenesis have been recently tested. Interestingly, PRS induces a life span reduction of hippocampal neurogenesis in male rats (Lemaire *et al.*, 2000, 2006) but not in females (Darnaudéry *et al.*, 2006), and a chronic agomelatine treatment increases hippocampal neurogenesis especially in the ventral part of the hippocampus of male rats (Morley-Fletcher *et al.*, 2011). As discussed above, PRS rats showed a reduced duration of slow wave sleep, an increased duration of rapid eye movement (REM) sleep, an increased number of REM sleep events and an increase in motor activity before the beginning of the dark phase of the light/dark cycle. In addition, adult PRS rats showed an increased expression of the transcript of the primary response gene, c-Fos, in the hippocampus just prior to the beginning of the dark phase. All these changes were reversed by a chronic oral treatment with agomelatine (2000 ppm in the diet). The effect of agomelatine on sleep was largely attenuated by treatment with the MT1/MT2 melatonin receptor antagonist, S22153, which caused PRS-like sleep disturbances on its own (Mairesse *et al.*, 2012). Moreover, reduced hippocampal levels of phosphorylated cAMP-responsive element binding protein (p-CREB), reduced hippocampal levels of mGlu2/3 and mGlu5 metabotropic glutamate receptors, and reduced neurogenesis in the ventral hippocampus of PRS rats were reversed by a 3- or 6-week treatment with agomelatine (40 mg/kg, i.p., once a day) (Morley-Fletcher *et al.*, 2011). Thus, antidepressants appear as a good probe to detect the neurobiological mechanisms that underlie the abnormalities induced by PRS. Enrichment environment was also able to reverse some of the abnormalities induced by PRS indicating that modification of late postnatal environment can constitute a valid alternative approach. These data strongly support the hypothesis that antidepressant drug impacts mechanisms that lie at the core of anxious/depressive disorders, thereby validating PRS as a model of depression.

- Construct validity. The stress-driven theory of mood disorders (Sapolsky, 1996; Kessler, 1997) suggests that a stress-induced model of depression such as the PRS model has good construct validity (Rosenwasser and Wirz-Justice, 1997). In this regard, early-life stress during critical developmental periods may induce, in some individuals, distinct and stable patterns of deregulations that are associated with altered emotional processing and heightened responsiveness to stress.

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Table 3: List of alterations induced by PRS (adapted from Darnaudéry and Maccari, 2008)

HPA axis dysfunction	Metabolic, immune dysfunctions	Behavioral dysfunctions
Hippocampal MR an GR receptors: ↘ of mRNA in the hippocampus of adolescent males: main effect in the CA3 (Van Waes et al., 2006); ↘ maximalbinding capacity in adult males (Henry et al., 1994; Maccari et al., 1995; Koehl et al., 1999) and females (Koehl et al., 1999). **Adrenal gland and weight:** ↘ at birth in males (Lesage et al., 2004; Mairesse et al., 2007a); ↗ in adolescent, adult, and 10 month-old males (Lemaire et al., 2000) and in 26 month-old females (Darnaudery et al., 2006). **Corticosterone secretion:** Basal levels: ↘ at birth in males (Lesage et al., 2004); ↗ at the end of the light phase in males and throughout the cycle in females (Koehl et al., 1997, 1999); ↗ in old males (Vallée et al., 1999). After novelty stress (corridor circular, restraint, elevated plus-maze): suppression of the stress hyporesponsive period in infant rats (Henry et al., 1994); ↘ of the sensitivity of the negative feedback of the HPA axis in adolescent, adult and 16 month-old males (Henry et al., 1994; Maccari et al., 1995; Barbazanges et al., 1996; Vallée et al., 1997, 1999; Morley-Fletcher et al., 2003a,b; Viltart et al., 2006). ↗ of the corticosterone response in 26 month-old females (Darnaudery et al., 2006). *After pharmacological stress (alcohol):* ↘ of the HPA response (CRH mRNA, POMC mRNA, corticosterone levels, ACTH levels) after an acute exposure to alcohol (1.5 g/kg, i.p.) in adolescent males (Van Waes et al., 2006).	**Body weight:** ↘ at embryonic day 21 in male and female fetuses (Lesage et al., 2004; Mairesse et al., 2007a,b); ↘ in adolescent males (Van Waes et al., 2006); ↘ in adult males (Vallée et al., 1996). **Glycemia:** ↘ at embryonic day 21 in male fetuses (Lesage et al., 2004); ↗ in 5 month-old adult males (Vallée et al., 1996) and in 24 month-old males (Lesage et al., 2004). ↗ after an oral glucose tolerance test in 24 month-old males (Lesage et al., 2004). **Feeding behavior:** ↘ in adult males (Vallée et al., 1996). ↗ more important after a fasting episode in 24 month-old males (Lesage et al., 2004). **Glucose transporter proteins in the placenta:** ↗ GLUT 1, ↘ GLUT 3, GLUT 4 (Mairesse et al., 2007a). **Immune function:** ↘ CD4+ cells, ↗ IL-1β levels in splenocytes and in brain, in 34–35 day-old adolescent males (Laviola et al., 2004). In 6 month-old males: ↗ CD8+; ↗ NK cells. ↗ proliferation of T lymphocytes, ↗ secretion of IFN-γ after stimulation in vitro by phytohemagglutinin-A (Vanbesien-Mailliot et al., 2007).	**Anxiety-depression:** ↘ exploration in the open arms of the elevated plus-maze (Vallée et al., 1997); ↗ reactivity to novelty in adult males (Deminiere et al., 1992; Vallée et al., 1997) and in adult females (Louvart et al., 2005). ↗ number of paradoxal sleep episodes in 3 month-old males (Dugovic et al., 1999). ↗ immobility in the forced-swim test; ↘ immobility after chronic antidepressant treatment in adult males (Morley-Fletcher, 2003a, 2004a). **Drug of abuse:** ↗ amphetamine self-administration in adult males (Deminiere et al., 1992); ↗ resistance to extinction to cocaine self-administration and ↗ cocaine-primed reinstatement (Kippin et al., 2007). ↗ locomotor response to amphetamine (Deminiere et al., 1992; Henry et al., 1995) and nicotine (Koehl et al., 2000) in adult males. ↗ motor impairments after MDMA (Ecstasy) in 30 day-old adolescent females (Morley-Fletcher et al., 2004b). Maintained high consumption levels after footshock in high-preferring adult females (Darnaudery et al., 2007). **Learning and memory:** ↘ spatial learning performances in 1 month-old and 3 month-old females (Wu et al., 2007) and in 4 month-old adult males (Lemaire et al., 2000) in the water maze (reference memory). ↘ spatial learning performances in the water maze (reference memory) in 26 month-old females (Darnaudery et al., 2006). ↘ spontaneous spatial recognition in juvenile (4 weeks) males and females (Gue et al., 2004) and in 15 and 21 month-old male (Vallée et al., 1999);↘ working memoryperformances in a radial maze in22 month-old males (Vallée et al., 1999).

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3 Depression and impairment in neurotransmission: targets of pharmacological treatment

3.1 Generalities on depression

Depressive disorders are among the most common human diseases: approximately 11% of world population experiences a time period of depression at least once in their life (Judd, 1995, Greenberg *et al.*, 2003). Depression involves several symptoms which occur together with a sufficient frequency and chronicity (more than two weeks) to constitute a recognizable clinical condition. The major features of depression are (i) a lack of motivation and inability to experience pleasure in anything (anhedonia), (ii) a loss of appetite, (iii) sleep disturbances (increased amount and decreased latency of paradoxical sleep), (iv) psychomotor retardation or agitation and (v) a phase shift in circadian activity patterns (DSM-IV criteria; Yadid *et al.*, 2000). Furthermore, depression has a significant problem of comorbidity with anxiety disorders and drug abuse (Zimmerman *et al.*, 2000; Schuckit, 2006). Although depression is believed to have a genetic component (Gershon *et al.*, 1987), environmental factors and, particularly stressful life events, may play a key role in inducing depressive episodes (Sapolsky, 1996; Kessler, 1997). Indeed, the stress-induced hyperactivity of the HPA axis is among one of the most relevant hormonal features observed, at least in a subpopulation of depressed subjects (de Kloet *et al.*, 2005; Holsboer *et al.*, 1984; 2010). This is characterized by hypersecretion of cortisol (Sachar *et al.*, 1973; Rubin *et al.*, 1987), resistance to dexamethasone test (Arana and Mossman, 1988) and increased levels of CRF in the cerebrospinal fluid (Nemeroff *et al.*, 1984). Recent findings in depressed patients have identified a reduction of 10-15% of hippocampal volume (Sapolsky 2000; Czeh and Lucassen, 2007). Postmortem studies have also shown a marked reduction of neurogenesis in the hippocampus of depressed individuals (Lucassen *et al.*, 2010).

A variety of therapeutic methods such as pharmacology, psychotherapy, electroconvulsive and magnetic therapies can be used to effectively treat depression but they all have a limited success (Nestler, 1998; Holsboer, 2001). Accordingly, only 60-65% of patients respond to the initial regimen and among those responding, less than half either reach remission or become symptom-free (Rosenzweig-Lipson *et al.*, 2007).

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3.2 Neurochemical alterations in mood-disorders

3.2.1 Monoamines

Barbiturates were the first drugs to treat anxiety, although during the past 3 decades, they were replaced by benzodiazepines (Harvey 1985; Dinan, 2006). However, benzodiazepines produce a number of adverse effects including sedation and cognitive impairments (Gudex, 1991; Barker *et al.*, 2004). The problem with the adverse effects of benzodiazepines led to the introduction of antidepressants for the treatment of anxiety, particularly selective serotonin reuptake inhibitors (SSRI; Ball *et al.*, 2005), which are presently employed in the treatment of a wide spectrum of anxiety disorders (Kent *et al.*, 1998).

The discovery of antidepressants led to the "monoaminergic hypothesis of depression", which assumed that the illness is due to the lack of serotonin and/or noradrenaline in the brain (Bunney & Davis, 1965; Schildkraut, 1965; Lapin & Oxenkrug, 1969). It was realized over 30 years ago that all the above-described effects of antidepressants occur instantly, while the antidepressant effect in humans requires several weeks of treatment (Oswald *et al.*, 1972), suggesting the involvement of adaptive changes in the therapeutic process.

The reduced 5-HT function is the most consistent biochemical marker that characterizes many depressed patients (Coppen *et al.*, 1972; Meltzer and Lowy, 1987). Indeed, a decrease in the uptake of 5-HT has been reported (Briley and Moret, 1993), as well as altered levels of 5-HT1A and 5-HT2 receptors in the cortex and in the hippocampus (Lesch *et al.* 1991; Hamon, 1995). An increase in serotonin availability in the synapse caused by antidepressant drugs supports the so-called "serotonergic theory of depression" (Blier *et al.*, 1990). Recent evidence suggests that not only tryptophan and consequent 5-HT depletion, but also induction of indoleamine 2,3-dioxygenase and the detrimental effects of tryptophan catabolites play a role in the pathophysiology of depression (Maes *et al.*, 2011). Sleep disturbances observed in depressed patients are also in agreement with the serotonergic theory, since an increase in the availability of 5-HT in the synapses caused by serotonin reuptake blockers can cause a decrease in the frequency of REM-sleep (Fornal and Radulovacki, 1983). Several studies have shown that noradrenaline is also implicated in the pathophysiology of depression. The β-adrenoceptor downregulation hypothesis of antidepressants action was introduced (Vetulani & Sulser, 1975), following experiments that demonstrated adaptive changes in α1- and α2-noradrenergic receptors and serotonergic 5-HT1 and 5-HT2 receptors (Vetulani *et al.*, 1981; Pilc & Vetulani, 1982; Vetulani *et al.*, 1983; Millan, 2005).

All antidepressants currently available on the market are believed to act principally on neurotransmission, either by inhibiting transmitter degradation (MAOIs) or by blocking the

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re-uptake of monoamines. Antidepressants produce synaptic changes with short-term administration and changes in receptors with long-term administration. Generally, all antidepressants have similar efficacy but are distinguished by their safety and side effects profiles. The SSRIs and the tricyclic antidepressants are among the most widely used antidepressants (DSM-IV, 1996; Yadid *et al.*, 2000).

SSRIs, such as fluoxetine (Prozac®) and paroxetine (Paxil®), increase the bioavailability of 5-HT in the synaptic cleft, including both the terminals and the bodies of neurones in all brain regions. Moreover, they have no significant interaction with other neurotransmitters and consequently cause considerably fewer side effects (Baldessarini, 1989). SSRIs are widely used for other disorders than depression, most notably obsessive-compulsive disorders and eating disorders. Their broad spectrum of clinical effects reflects the widespread involvement of the 5-HT system in many biological functions. With this class of antidepressants, the starting dose is usually the same as the one the one used for a full therapeutic dosage.

Agomelatine is a specific agonist of the MT1 and MT2 receptors of melatonin (Ying *et al.*, 1996) and it is also a selective antagonist of the 5-HT2C receptors of serotonin (Millan *et al.*, 2003). As an agonist, agomelatine mimics the effects of melatonin on different systems (Ying *et al.*, 1996; Martinet *et al.*, 1996). For example chronic administration of agomelatine in rat is able to resynchronize an altered circadian rhythm (Van Reeth *et al.*, 1997; Mairesse *et al.*, 2012) or reverse the anxious/depressive phenotype in animal model of depression (Maccari and Nicoletti, 2011; Morley-Fletcher *et al.*, 2011; Rainer *et al.*, 2012). In the rat model of chronic moderate stress, chronic treatment with agomelatine produces the same effects of imipramine and fluoxetine in sugar intake (Papp *et al.*, 2003); also, agomelatine influences the immobility time comparable to those of the imipramine in the model of the forced swimming in rat, (Bourin *et al.*, 2004) and, recent studies point out its efficacy in the rat model of learned helplessness (Bertaina-Anglade *et al.*, 2006). Interestingly, agomelatine is effective in the corticosterone mice model of anxiety/depression (Rainer *et al.* 2012). Several clinical studies have demonstrated the clinical efficacy of agomelatine in patients with major depressive disorder (Olié and Kasper 2007; Goodwin *et al.*, 2009) as well as its similar effectiveness to that of classical antidepressants (de Bodinat *et al.*, 2010).

Tianeptine (Stablon®) is among one of the most recent tricyclic antidepressants. It has a well-documented therapeutic activity (Mennini and Garattini, 1991; Delbende *et al.*, 1994; Dziedzicka-Wasylewska *et al.*, 2002), although devoid of the classic action of antidepressants on central monoamine activities. In contrast to most antidepressants,

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tianeptine decreases extracellular serotonin levels and exhibits no affinity for neurotransmitter receptors (Wagstaff *et al.*, 2001). Like the SSRIs, tianeptine produces less adverse effects as compared to classic tricyclics and has a low propensity for abuse. Moreover, there are increasing evidences that this antidepressant can prevent or even reverse stress-induced changes in cerebral morphology that are often associated with stress-related psychiatric disorders (Watanabe *et al.*, 1992; McEwen *et al.*, 1997; Magarinos *et al.*, 1999; Malberg *et al.*, 2000; Czeh *et al.*, 2001). The antidepressant effects of tieneptine cannot be entirely attributed to the monoaminergic action, as recent evidence suggests that this antidepressant has also a strong impact on the glutamatergic system (McEwen, 2010). Accordingly, Pittaluga and coworkers (2007) have shown that classical antidepressants may act through glutamate ionotropic receptors to mediate amine release in the hippocampus. Antidepressants were shown to depress binding (Nowak *et al.*, 1993; Paul *et al.*, 1994), expression (Bartanusz *et al.*, 1995) and function (Pallotta *et al.*, 2001; Paul and Skolnick, 2003) of glutamatergic NMDARs. Furthermore, selective NMDAR antagonists exhibit antidepressant-like activity (Skolnick *et al.*, 2009). AMPARs also seem to be affected by antidepressant treatment; some authors reported their expression to be up-regulated following chronic antidepressants (Martinez-Turrillas *et al.*, 2005; Choudary *et al.*, 2005 and Barbon *et al.*, 2006).

3.2.2 Glutamate/GABA balance

Compelling evidence describes alterations in the glutamate levels of blood and cerebrospinal fluid (CSF) in patients suffering of major depression. Kim and coworkers (1982) reported that serum levels of glutamate in patients with depression were significantly higher than those of healthy controls. Furthermore, Altamura and coworkers (1993) reported that plasma levels of glutamate in patients with mood disorders were significantly higher than those in the control group. Other groups have also confirmed that the levels of glutamate are increased in patients with depression (Mauri *et al.*, 1998; Mitani *et al.*, 2006). In addition, there is a positive correlation between plasma glutamate levels and severity of depressive symptoms in patients with depression (Mitani *et al.*, 2006). Hashimoto and coworkers (2007) reports increased levels of glutamate in the frontal cortex, suggesting an abnormality of glutamatergic neurotransmission in the pathophysiological features of depression. Also, several studies have demonstrated differences related to glutamate receptors (AMPA, kainate, and NMDA receptors) in postmortem brain samples from individuals with depression. An *in situ* hybridization study demonstrated that mRNA levels of the NR2A and NR2B subunits of the NMDA receptors and the GluR1, GluR3, and GluR5 subunits of the AMPA receptors in the perirhinal cortex, but not in the hippocampus or entorhinal cortex, in patients with depression

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were significantly lower than those of a control group (Beneyto *et al.*, 2007). Furthermore, it has been reported that levels of the NR2A and NR2B subunits of the NMDA receptors are decreased in the prefrontal cortex (Brodmann's area 10) of patients with depression (Feyissa *et al.*, 2010). A noninvasive *in vivo* proton magnetic resonance spectroscopy study revealed increased levels of glutamate or the ratio of glutamate to GABA in the occipital cortex of depressed patients (Sanacora *et al.*, 2004).

The roots of a 'glutamate hypothesis of depression' can be traced back to the observations that exposure to inescapable but not escapable shock disrupted hippocampal NMDA-dependent LTP (Shors *et al.*, 1989). Contemporarily, early findings showed that NMDA receptors antagonists possessed antidepressant-like action (Trullas and Skolnick, 1990). Glutamate is also the precursor of free GABA in GABAergic terminals and comes from two different sources (the Kreb's cycle in glia cells and glutamate in nerve terminals). GABA in GABAergic terminals is formed from glutamate in an enzymatic reaction mediated by glutamic acid decarboxylase (GAD)-65/67, using pyridoxal phosphate as cofactor (Brambilla *et al.*, 2003) (Fig.16). Very recently, reduced levels of GAD-67 in the dorsolateral prefrontal cortex (Brodmann's area 9) of patients with depression were demonstrated. This led to a dysfunction of the glutamate-GABA system in the neurobiology of depression (Karolewicz *et al.*, 2010). These findings suggest that disturbances in glutamate/GABA-mediated synaptic neurotransmission in the brain might be implicated in the pathophysiology of depression (Krystal *et al.*, 2002; Hashimoto, 2009; Hasler, 2009).

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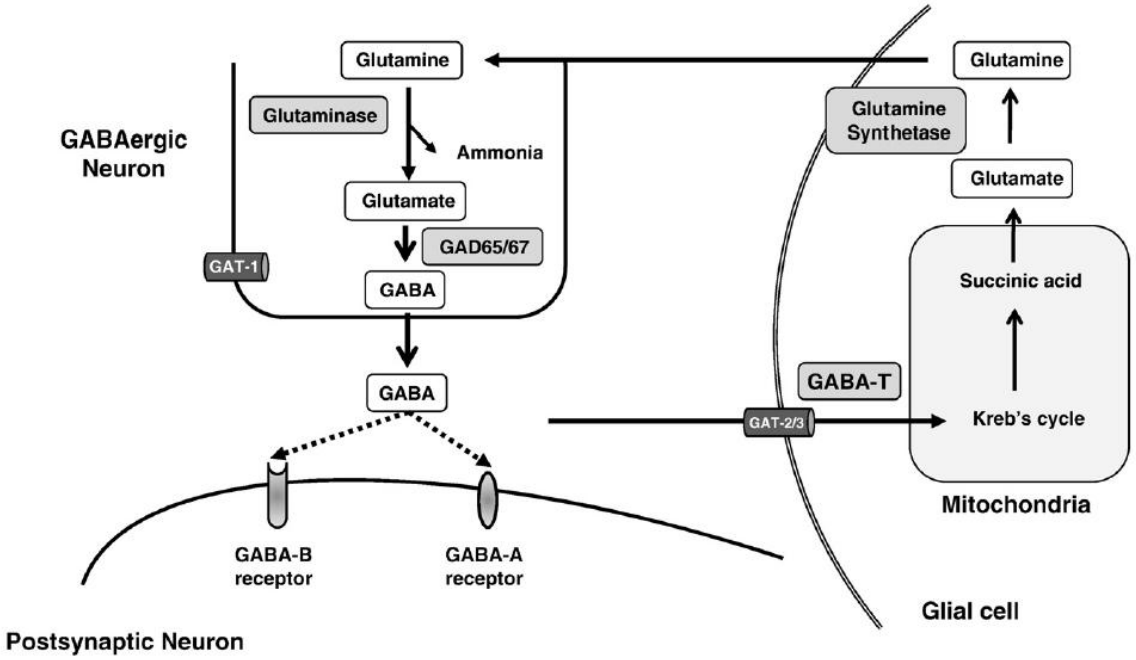

Figure 16: Glutamate is the precursor of GABA in GABAergic terminals. The enzyme glutamic acid decarboxylase (GAD) forms GABA from glutamate. After being released into the synapses, GABA binds to GABA receptors ($GABA_A$ and $GABA_B$) on the postsynaptic neurons. Then, GABA is inactivated by reuptake mediated by GABA transporters (GAT-1 and GAT-2/3) into the presynaptic terminals or into glial cells, where it is metabolized by GABA-transaminase (GABA-T). Glutamate also comes from the Kreb's cycle in the mitochondria of glial cells. (from Hashimoto, 2011)

As mentioned in the first section of the Introduction, stress is a major risk factor for depressive syndromes and this can be associated to changing in glutamate release (Bagley and Moghaddam, 1997; Lowy *et al.*, 1995; Reznikov *et al.*, 2007). Musazzi and coworkers (2010) reported that, in prefrontal/frontal cortex, depolarization-dependent release of glutamate was selectively up-regulated by acute stress relative to GABA release, and that chronic treatment with antidepressants (desipramine, fluoxetine or venlafaxine) completely abolished the stress-induced up-regulation of glutamate release, suggesting that this may be a relevant component of the therapeutic action of antidepressants (Musazzi *et al.*, 2010). Preclinical findings have shown that that olfactory bulbectomy, an established animal model of depression, caused alterations in mGluR subunits in the mouse hippocampus, and that these alterations were reversed by antidepressant treatment (Wierońska *et al.*, 2008). Two studies using postmortem brain samples demonstrated an elevated level of mGluR2/3 protein (Feyissa *et al.*, 2010) and a reduction of mGluR5 protein (Karolewicz *et al.*, 2010) in the prefrontal cortex of patients with depression. Nowadays, the targeting of mGluR emerged an attractive approach for the development of novel antidepressant drugs. For example, the highly subtype-selective mGluR1 antagonist (JNJ16567083 (R176898), 3-ethyl-2-methyl-quinolin-6-yl-(4-methoxy-cyclohexyl)-methanonemethanesulfonate), has

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antidepressant-like effects in the rat forced-swim and the mouse tail-suspension tests (Belozertseva *et al.*, 2007). Also, the selective mGluR5 antagonists, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and the more selective and metabolically stable analog 3-[(2-methyl-1,3-thiazol-4-yl)ethylnyl]-pyridine (MTEP), have antidepressant-like effects in the mouse tail-suspension test, an animal model of depression (Belozertseva *et al.*, 2007; Tatarczyńska *et al.*, 2001; Palucha *et al.*, 2005). Group II mGluRs agonists and antagonists also possess an antidepressant activity (Palucha and Pilc, 2007; Sanacora *et al.*, 2008; Hashimoto, 2011). Accordingly, the mGluR2/3 antagonists, including LY341495 and MGS0039, have been shown to exhibit antidepressant-like effects in animal models of depression. Both LY341495 (0.1–3 mg/kg, i.p.) and MGS0039 (0.3–3 mg/kg, i.p.) had dose-dependent antidepressant-like effects in the rat forced-swim test and in the mouse tail-suspension test (Chaki *et al.*, 2004, Ago *et al.*, 2012). Both agonists and antagonists of group III mGluRs also show antidepressant-like action (Tatarczynska *et al.*, 2002; Palucha *et al.*, 2004; Suzuki *et al.*, 2007, Hashimoto, 2011).

More recently, the "glutamatergic hypothesis of depression" has been integrated with results from different fields including intracellular signaling/mechanisms of gene expression, neurotrophic mechanisms, neurogenesis, synaptic function/plasticity and remodeling of neuronal cells/circuitry (Pittenger and Duman, 2008; Racagni and Popoli, 2008; Sanacora *et al.*, 2008). These findings are preferentially referred to the "neuroplasticity hypothesis of depression". Data on hippocampal neurogenesis support this theory by showing that neurogenesis is required for the behavioral effects of antidepressants (Santarelli *et al.*, 2003). Antiglucocorticoids used in rat model of depression have shown an interesting efficacy in normalizing neurogenesis in the dentate gyrus (Mayer *et al.*, 2006; Oomen *et al.*, 2007), as well as LTP (Kugers *et al.*, 2006) and calcium current amplitude in the CA1 region (Karst and Joëls, 2007). A number of studies has proposed that the downstream neural adaptation (for example BDNF pathway) rather than the elevation in synaptic monoamine levels may be responsible for the therapeutic effect of antidepressants (Duman *et al.*, 1997; Nestler *et al.*, 2002; Hashimoto 2010). However, the link between antidepressant activity and stimulation of neurogenesis is not completely unequivocal (Krugers *et al.*, 2010). Indeed, not all antidepressants stimulate neurogenesis, and both neurogenesis-dependent and neurogenesis-independent mechanisms of antidepressant action exist (Sahay and Hen, 2007; David *et al.*, 2009).

The aim in developing effective antidepressants is to achieve a fast onset of action and to enhance clinical efficacy while reducing side effects at the same time. Neurobiological and neuroanatomical studies indicate the importance of changes in both noradrenergic and

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serotonergic systems for the success of an antidepressant treatment (Van Praag *et al.*, 1990; Delgado *et al.*, 1993). Limbic structures are undoubtedly the main target for all the antidepressant approaches that we have summarized above. Unfortunately, the beneficial effects of the drugs are evident only after several weeks of continued administration (Blier and de Montigny, 1994; Nestler, 1998). Also, there is a great incidence in the relapse and the recurrence of depressive episodes after the interruption of treatment, leading to consider depression as a chronic illness that requires a prolonged treatment (Buller and Legrand, 2001). This suggests that novel therapeutic advents are needed to improve the treatment of depression.

The stress theory of mood disorders suggests that stress induced-models of depression have good construct validity (Rosenwasser and Wirz-Justice, 1997; Willner, 1997) and, therefore, they represent an interesting approach for the investigation of new mechanisms that lie at the core of depressive disorders and antidepressants treatment.

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AIM OF THE THESIS

As widely described in the Introduction, insults, during critical periods of life, result in the programming of a maladaptive phenotype that persists during the life span (Lupien *et al.*, 2009; Franklin *et al.*, 2012). In particular, early life stress is a major risk factor for anxious/depressive-like syndromes in adulthood. In rats, the model of prenatal restraint stress (PRS) nicely recapitulates the key-features of depressive disorders, thereby meeting the *criterium* of face validity (Maccari *et al.*, 1995; Darnaudéry and Maccari, 2008; Morley-Fletcher *et al.*, 2011).

Although depression was firstly linked to lack of monoamine bioavailability within serotonergic and noradrenergic synapses (Bunney and Davis 1965; Schildkraut, 1965; Lapin and Oxenkrug, 1969), mounting evidence suggests that glutamatergic system exerts a central role in the pathophysiology of mood and depressive disorders. Thus, beyond the monoaminergic hypothesis of depression, novel pharmacological approaches insist on glutamate-based therapy, rising the interesting possibility that classical and novel antidepressant drugs may "correct" the pathological phenotype *via* the glutamatergic system (Palucha and Pilc, 2007; Skolnick *et al.*, 2009; McEwen, 2010; Hashimoto, 2011; Sanacora *et al.*, 2012). Despite several studies have investigated the glutamate-stressed synapses in many brain regions, including prefrontal cortex, amygdala and striatum (Fumagalli *et al.*, 2009; Mozhui *et al.*, 2010; Farley *et al.*, 2012; Popoli *et al.*, 2012), little is known on the influence of glucocorticoids-related stress on the glutamatergic neurotransmission in hippocampus.

The aim of the present thesis was **to investigate the glutamatergic hypothesis of depression in the rat model of PRS, by focusing on the neuroadaptation within the hippocampal circuit.**

This manuscript is developed on three main axes that can be summarized as following.

Most of the abnormalities in synaptic machinery and plasticity in the hippocampus of PRS rats are found in the ventral hippocampus, the specific portion of the hippocampus that encodes memories related to stress and emotions (Zuena *et al.*, 2008). Thus, i**n CHAPTER ONE**, we wanted to establish the role of ventral hippocampal glutamate neurotransmission in the pathophysiology of the anxious/depressive profile induced by PRS. To this purpose, we assessed two main questions that, respectively, result in two sections:

a) we performed a proteomic analysis in the hippocampus of both control and PRS rats, in order to point out the main pathways and proteins, affected by PRS;

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b) we investigated the glutamatergic neurotransmission in the hippocampus and we hypothesized that the extent of glutamate impairment could be related to the anxious/depressive profile of PRS rats;

Most of the changes induced by PRS, suchs as reduction in neurogenesis and increased anxious/depressive-like behavior, are totally reversed by chronic treatment with antidepressants (Morley-Fletcher *et al.*, 2004a; 2011). We know that antidepressants, at least in part, act to "normalize" the glutamatergic system (Bonanno *et al.*, 2005; McEwen, 2010). Hence, **in CHAPTER TWO,** we wanted to assess whether antidepressant drugs may exert their role on glutamate system, thereby reversing the glutamate-related alterations described in Chapter ONE. Thus, we extended our investigation on the neurobiological action of both agomelatine – a novel antidepressant drug that behaves as a mixed MT1/MT2 melatonin receptor agonist/5-HT2C receptor antagonist – and fluoxetine, a classical SSRI antidepressant.

In CHAPTER THREE we moved from the evidence that toxin-induced damage of the ventral hippocampus restrains the motor response to antipsychotic drugs, suggesting that the ventral hippocampus modulates the basal ganglia motor circuit (Lipska and Weinberger, 1993). Thus, we analyzed the role of the prenatally programmed "stressed hippocampus" in the putative control of the pharmacodynamics of mesocorticolimbic-related structures, such as striatum. We then opened a window on motor disorders by studying the influence of PRS on haloperidol-induced catalepsy, a detrimental consequence of the action of classical antipsychotics on striatal dopaminergic D2 receptors.

Finally, data presented within the Results section will describe a point by point analysis to the aforementioned questions, aimed to strength the findings that PRS is an attractive animal model to study depression and mood disorders.

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RESULTS

-CHAPTER ONE-

The involvement of neurotransmission in mood disorders: a focus on glutamatergic system

1. Proteomic characterization in the hippocampus of prenatally stressed rats

Preface

The human genome contains an estimated 20,500 genes (Clamp *et al.*, 2007). Between one-third to one-half of these genes are preferentially expressed in the brain (Colantuoni *et al.*, 2000). These genes are involved in neuronal/glial development, pathfinding, growth, repair, maintenance and, in some instances, they might be involved in regulating the aging process (Kenyon, 2010). Recent major advances in proteomics offer us powerful tools to evaluate global fluctuations in gene and protein expression in the central nervous system. Proteomics was a term first coined in the mid-1990s (Wilkins *et al.*, 1996) in the context of the field of "genomics," and like transcriptomics, which assesses global mRNA, proteomics is viewed as a high throughput, discovery (hypothesis-generating) research method. Clinical neuroproteomics is part of the proteomics field concerned with finding biomarkers of central nervous system diseases or drug response to improve our understanding of the pathophysiology of brain disorders. Other aspects of the neuroproteomics field focus on understanding protein–protein interactions, organization, and networks, whereas others focus on characterizing and describing whole proteomes in the context of the central nervous system. In proteomics, there are two main methods that are viewed as sufficiently reproducible and high throughput: the traditional, gel-based, two-dimensional gel electrophoresis (2-DE) method coupled to mass spectrometry (MS) and the more recently developed, non-gel-based liquid chromatography/mass spectrometry (LC/MS)-based profiling. MS has revolutionized the field of proteomics because of its ability to identify proteins, by measuring the molecular mass-to-charge (m/z) ratio of ions (i.e., molecules or peptides that have been electrically charged) (Morelle and Michalski, 2005; English *et al.*, 2011; Aebersold and Goodlett, 2001). Much of the work in the field of clinical neuroproteomics has been carried out in neurodegenerative disorders such as Alzheimer's disease (Song *et al.*, 2009; Hwang *et al.*, 2009). Application of these techniques to

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neurodegenerative disorders is especially advantageous because, despite decades of ‘‘mechanism’’- or ‘‘pathway’’ -based pursuits, the real number of protein implicated in these diseases remains largely unknown (Lefebvre *et al.*, 2010; Cookson, 2005; Moore *et al.*, 2005; Thomas and Beal, 2007; Arakawa, Kita, and Niikura, 2008; Siddique and Siddique, 2008). Most recently, the use of quantitative proteomics to investigate disease-specific protein signatures holds great promise to improve the understanding of psychiatric disorders, by the identification relevant biomarkers (Uys *et al.*, 2006; Filiou *et al.*, 2011; English *et al.*, 2011). Interestingly, in humans, frontal cortex and nucleus accumbens from depressed patients have been subjected to proteomic analyses, revealing differentially expressed proteins (Beasley *et al.*, 2006; Johnston-Wilson *et al.*, 2000). Also, proteomic-based studies are beginning to elucidate interesting biomarkers in animal models of depression (Mu *et al.*, 2008; Martins-de-Souza *et al.*, 2010). Accordingly, Mu and coworkers have identified 27 differentially expressed proteins with a role in neurogenesis and oxidative metabolism in the hippocampus of a chronic stress rat model of depression (Mu *et al.*, 2007).

In this section we have performed a proteomic analysis in the hippocampus of adult male PRS rats, by 2-D electrophoresis and MS, in order to identify interesting bio-molecular pathways and proteins that may lie at the core of the pathological programming triggered by early life stress.

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Proteomic characterization in the hippocampus of prenatally stressed rats

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ABSTRACT

Rats exposed to early life stress are considered as a valuable model for the study of epigenetic programming leading to mood disorders and anxiety in the adult life. Rats submitted to prenatal restraint stress (PRS) are characterized by an anxious/depressive phenotype associated with neuroadaptive changes in the hippocampus. We used the model of PRS to identify proteins that are specifically affected by early life stress. We therefore performed a proteomic analysis in the hippocampus of adult male PRS rats. We found that PRS induced changes in the expression profile of a number of proteins, involved in the regulation of signal transduction, synaptic vesicles, protein synthesis, cytoskeleton dynamics, and energetic metabolism. Immunoblot analysis showed significant changes in the expression of proteins, such as LASP-1, fascin, and prohibitin, which may lie at the core of the developmental programming triggered by early life stress.
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1. Introduction

The low discordance of depression between monozygotic twins and the slow progress in identifying genetic risk factors suggest that epigenetic changes largely contribute to the individuals' vulnerability to major depressive disorder [1]. Both human and animal studies suggest that exposure to stressful events during critical periods of brain development triggers an epigenetic programming leading to low resilience to stress in the adult life [2–8]. Abnormalities of synaptic transmission and plasticity in the hippocampus represent an integral part of this epigenetic program. For example, early life stress resulting from low maternal care in rodents causes a permanent reduction in the length of dendritic branching and the number of dendritic spines associated with an impairment of synaptogenesis and long-term potentiation in the hippocampus [9–11]. This fits nicely with the clinical evidence that poor parental care can compromise cognitive development [12,13].

Rats exposed to prenatal restraint stress (PRS) develop long-lasting biochemical and behavioral changes that likely reflect the induction of a pathological epigenetic programming [14,15], and therefore represent a model that meets the criterium of *construct validity* because it replicates environmental factors implicated in the etiology of depression and other stress-related disorders [1]. Alterations induced by PRS comprise a dysfunction of the hypothalamo-pituitary-adrenal (HPA) axis which is reversed by cross fostering at birth [16], a generalized disorganization of circadian rhythms and the sleep–wake cycle, an age-dependent impairment in spatial

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learning, a lifelong reduction of hippocampal neurogenesis, and changes in the levels of brain-derived neurotrophic factor (BDNF), cyclic-AMP responsive element binding protein (CREB), and group-I and group-II metabotropic glutamate receptors in the hippocampus [16–20]. Remarkably, some of these changes are reversed by chronic antidepressant treatment [21–23]. Hence, the rat model of PRS is particularly valuable for a systematic analysis of hippocampal proteins that are the product of the epigenetic programming leading to a low resilience to stress and to an anxious/depressive phenotype in the adult life. Here, we examined the protein expression profile in the hippocampus of adult rats exposed to PRS by using a proteomic approach based on the use of two-dimensional electrophoresis coupled with mass spectrometry.

2. Materials and methods

2.1. Animals

Nulliparous female Sprague–Dawley rats, weighing approximately 250 g, were purchased from a commercial breeder (Harlan). Animals were housed at constant temperature (22±2 °C) and under a regular 12 h light/dark cycle (lights on at 8.00 a.m.). Pregnant females were randomly assigned to stressed or control groups (n=12 per group).

2.2. Stress protocol

Animals were subjected to PRS according to our standard protocol [16,21]. From day 11 of pregnancy until delivery, pregnant female rats were subjected to three stress sessions daily (45 min each), during which they were placed in transparent plastic cylinders and exposed to bright light. Only male offspring from litters containing 10–14 pups with a comparable number of males and females were used for the experiments. All experiments followed the rules of the European Communities Council Directive 86/609/EEC. The prenatal stress procedure was approved by the local ethical committee.

2.3. Protein sample preparation and 2D analysis in the hippocampus

Hippocampi of PRS and control rats (n=6/group) were rapidly dissected, frozen on dry ice and stored at −80 °C. Samples were then homogenized with a glass/Teflon homogenizer at a concentration of 10% (w/v) in a solubilizing solution containing: 7 M urea (Sigma-Aldrich, St. Louis, MO, USA), 2 M thiourea (Fluka, Buchs, Switzerland), 40 mM Tris (Sigma-Aldrich), 2% CHAPS (Fluka), and Complete™ protease inhibitor (Roche, Basel, Switzerland). Samples were sonicated three times for 10 s on ice with an ultrasonic processor with probe (Ultrasonic 2000, Dynatech Laboratories Inc., Chantilly, VA, USA). The extract was centrifuged at 1000 *g* and the pellet discarded. An aliquot of this supernatant was used to measure protein concentration by the Bradford method [24]. 100 μg of proteins was separated by 2D electrophoresis following a step of passive rehydration on 18 cm immobilized pH gradient strips (IPG; non-linear pH gradient of 3–10, GE Healthcare, France) overnight. Focusing was carried out for 24 h at 20 °C for a total of 100,000 Vh on a pHaser isoelectric focusing system (Genomic solutions, Cambridgeshire, UK). The focused IPG strips were equilibrated for 20 min with gentle shaking in an equilibration solution (6 M urea, 2% SDS, 375 mM Tris pH 8.8, 30% glycerol) containing 1% DTT, and then with 2% iodoacetamide. The strips were applied to 10% SDS polyacrylamide gels using the Investigator System (Genomic Solutions), and finally, 2D gels were silver-stained. For each animal, 2-D gel electrophoresis was performed in triplicates for a total of 36 gels.

Electrophoresis images of gels were digitized using the GS-710 densitometer system (Bio-Rad). 2D gel analysis was carried out with Progenesis SameSpots software (Nonlinear Dynamics, Ltd).

2.4. Statistics

The aligned images were grouped into their corresponding PRS or control group and the statistically ranked list of spots was evaluated in the review stage of the SameSpots software. Protein levels were evaluated as volumes (spot area × optical density) for the protein spots matched among gels. Spot volume for valid spots was normalized to total density for each gel. Our criteria for evaluation of protein spots were based on an ANOVA p-value<0.05 as calculated with the built-in statistical tools in the software and a minimum of 1.5-fold intensity (normalized volume) in protein content between PRS and control animals. Then, only spots within the range of 1.5–3.5 fold change were cut out (24 spots) and processed for LC–MS/MS analysis.

2.5. Protein identification with LC–MS/MS

The gel with the highest spot intensity was selected for manual excision for evaluation by mass spectrometry. Spots of interest were carefully cut from the gel, destained in a solution containing 1.6% thiosulfate and 1% potassium ferricyanide, extensively washed in water, and then submitted to *in-gel* trypsin digestion. Briefly, after reduction and alkylation, trypsin digestion was performed overnight at 37 °C in 25 mM ammonium bicarbonate (porcine mass spectrometry grade MSG-Trypsin; G-Biosciences, Agro-Bio, La Ferté St Aubin, France). Peptides were extracted in 45% acetonitrile/45% water/10% trifluoroacetic acid (TFA) (v/v/v) and then dried in a speed-vac (Eppendorf) before nano-high pressure liquid chromatography (HPLC)–MS/MS analysis. NanoLC-NanoESI–MS/MS analyses were performed either on an ion trap mass spectrometer (LCQ Deca XP+, Thermoelectron, San Jose, CA) equipped with a nano-electrospray ion source coupled to a nano-flow high-pressure liquid chromatography system (LC Packings Dionex, Amsterdam, The Netherlands) as previously described [25], or on an hybrid quadrupole time-of-flight mass spectrometer (Q-Star, Applied Biosystems, Foster City, California, USA) equipped with a nano-electrospray ion source coupled with a nano HPLC system (LC Packings Dionex, Amsterdam, The Netherlands). Peptidic samples were dissolved in 5 μL 95% H2O/5% ACN / 0.1% HCOOH (v/v/v) (solvent A) and were injected into the mass spectrometer using the Famos auto-sampler (LC Packings Dionex, Amsterdam, The Netherlands). Samples were desalted and concentrated on a reserved-phase precolumn of 0.3 mm i.d.×5 mm (Dionex) by solvent A delivered by the Switchos

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pumping device (LC Packings Dionex), at a flow rate of 10 μL/min for 3 min. Peptides were then separated on a 75 μm i.d.×15 cm C18 Pepmap column (Dionex). The flow rate was set at 200 nL/min. Peptides were eluted using a 0% to 35% linear gradient of solvent B (25% H2O/75% ACN/0.1% HCOOH) in 80 min then a 35% to 100% linear gradient of solvent B in 10 min and finally 100% of solvent B was maintained for 5 min. Coated electrospray needles were obtained from New Objective (Woburn, Massachusetts, USA). The spray voltage was 1.65 kV. The mass spectrometer was operated in the positive ion mode. Data acquisition was performed in a data-dependent mode consisting of, alternatively, a full-scan MS over the range *m/z* 300–2000, and a full-scan MS/MS of the ion selected over the range *m/z* 50–2000 in an exclusion dynamic mode (the most intense ion is selected and excluded for further selection for a duration of 30 s). MS/MS data were acquired using a mass tolerance of 50 mmu and the collision energy was automatically fixed by the device. For the automated database search of fragment ion spectra, the Analyst QS software and Mascot dll script were used and final database searching was performed using Mascot software (Matrix Science London, UK, MS/MS ion search module), in the Swiss-Prot database (Sprot 0411, 525,207 sequences). Search parameters were as follows: Rattus as the taxonomic category, 100 ppm tolerance for the parent ion mass and 50 mmu for the MS/MS fragment ions, one missed cleavage allowed, carbamidomethylcysteine as a fixed modification, and methionine oxidation as a possible modification. Only proteins with a significant Mascot score were taken into consideration and reported after manual verification of the fragmentation spectra.

2.5. *Western blot validation of identified proteins*

A separate set of animals was used for immunoblotting experiments. Four to six animals per group were analyzed in duplicate. Rats were killed by decapitation and brains rapidly removed; hippocampi (dorsal and ventral where described) were dissected and stored at −80 °C until homogenization.

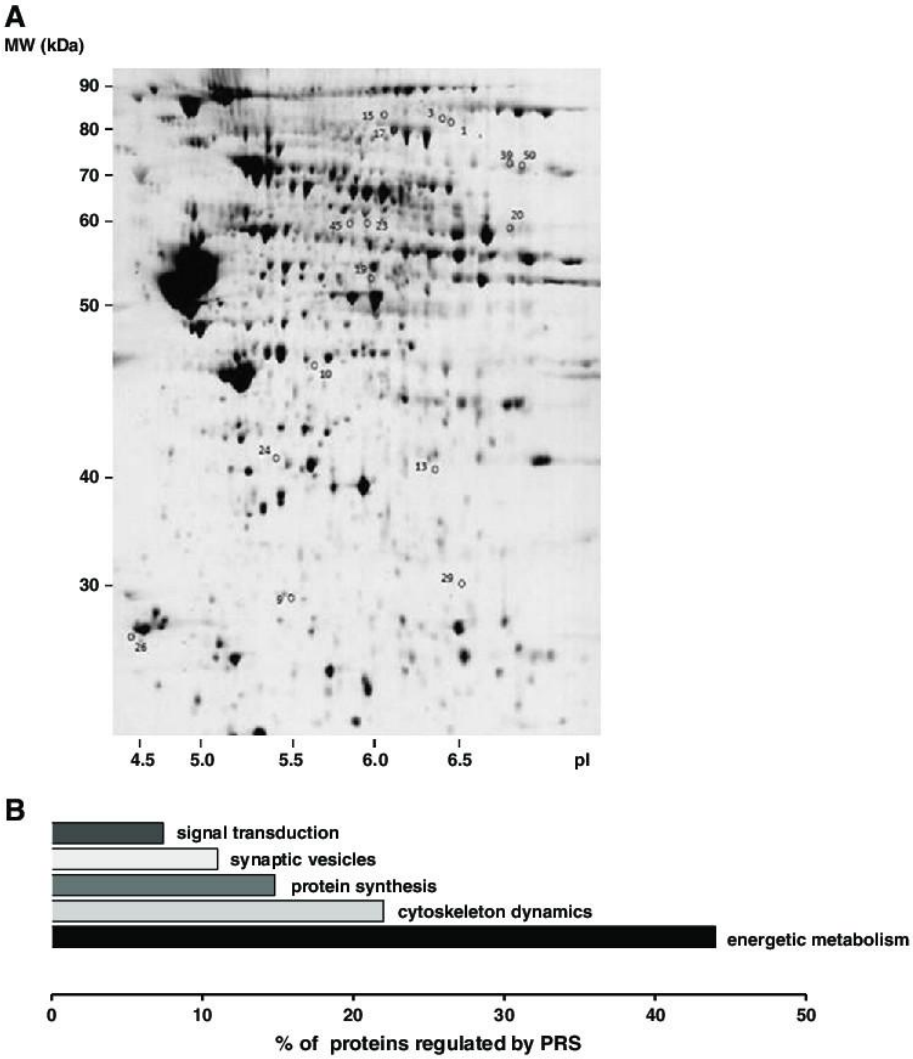

Fig. 1 – (A) Representative 2D gel image with spots of proteins listed in Table 1 in the 3–10 pH range. (B) Functional clustering of the identified proteins regulated by PRS in the adult hippocampus.

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Tissues were homogenized at 4 °C with a TissueRuptor (Quiagen) in lysis buffer (pH 7.4) containing: 320 mM Sucrose, 5 mM Hepes, 500 mM sodium fluoride, 10% SDS and phosphatase/protease inhibitor (Sigma). BCA assay was used to determine protein concentration. Lysates were resuspended in laemli reducing buffer and 25 μg of each sample was first separated by electrophoresis on 8–12% SDS-polyacrylamide gels and suddenly later transferred to nitrocellulose membranes (Biorad). Transferring was performed at 4 °C in a buffer containing 35 mM TRIS, 192 mM glycine and 20% methanol.

The following primary antibodies were used to detect the relevant proteins: anti-Prohibitin (Thermo Scientific; 1:1000), anti-LASP-1 (Millipore; 1:1000), anti-Fascin (Santa Cruz; 1:2000), anti-Transferrin (AbCam; 1:5000), anti-β-Actin (Sigma; 1:80000). Secondary antibodies directed against rabbit or chicken were used at 1:10,000 dilution. Densitometric analysis was performed with Quantity One software (Bio-Rad) associated to a GS-800 scanner. A ratio of target to β-Actin was determined and these values were compared for statistical significance with the Student's *t*-test.

3. Results

3.1. *PRS altered the hippocampal proteome*

To identify novel proteins modified by PRS we compared the proteome in the hippocampus of adult male PRS and control rats. Analysis of two-dimensional electrophoresis patterns by using Progenesis SameSpots Software revealed that the densities of 24 spots were significantly different ($p<0.05$) between control and PRS rats.

Among them, 18 spots with a 1.5–3.5 fold change shown on a gel in Fig. 1A, were unambiguously identified as known proteins by nanoLC–tandem mass spectrometry. A total of 26 different identified proteins were thus sorted into the following 5 groups based on their biological function: (i) signal transduction; (ii) synaptic vesicles; (iii); protein synthesis (iv) cytoskeleton dynamics; and (v) energetic metabolism (Fig. 1B). These proteins are listed in Table 1 and Supplemental Table 1. We identified up to 6 different proteins per regulated spot in some cases, due to possible overlapping protein spots in the wide pH 3–10 range. The image analysis identified spots 3 and 9 as being up-regulated by PRS whereas all the other spots were found to be down regulated. We could identify mainly soluble and cytosolic proteins. It is therefore likely that many other changes remained undetected, particularly those involving low abundant proteins, or more hydrophobic and high molecular weight proteins.

3.2. *Immunoblotting validation of proteomic data*

In order to confirm proteomic data in the hippocampus, the expression of proteins previously identified within the regulated spot were examined by immunoblot analysis in a separate set of animals (Fig. 2). We found that PRS decreased the expression of Lasp-1 (spot no. 13; $F(1,8)=7.73$, $p<0.05$) and increased the expression of transferrin (spot no. 3; $F(1,8)=10.21$, $p<0.05$), prohibitin (spot no. 9; $F(1,8)=13.19$, $p<0.05$), and fascin (spot no. 19; $F(1,8)=6.16$, $p<0.05$). The increase in the expression of fascin could appear discordant with the general down regulation profile of spot 19, as revealed by MS/MS analysis. However, fascin accounts for the 30% only of the peptides mixture if we take into account the fifteen different sequences of peptides identified within the spot. The decrease in intensity of spot 19 observed in the comparative analysis of 2D-gels may thus come from the other identified candidates.

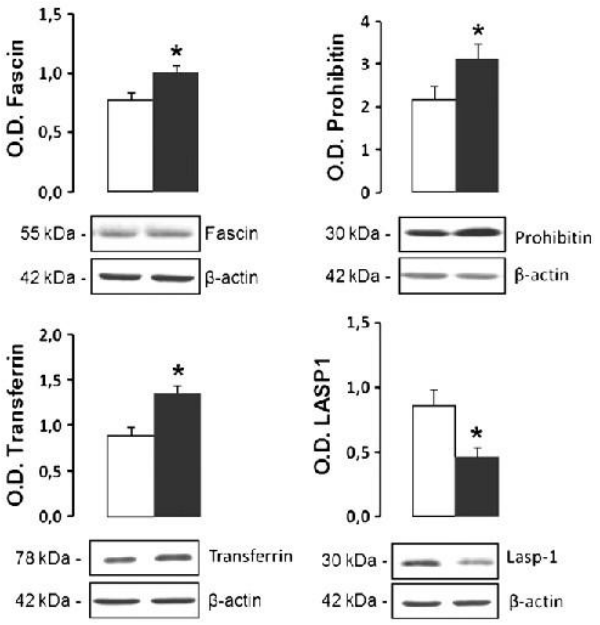

Fig. 2 – Immunoblot analysis of fascin, prohibitin, transferrin, and LASP-1 in the hippocampus of control and PRS adult rats. Values are means + S.E.M. of 6 biological replicates. *$p<0.05$ vs. controls.

4. Discussion

This study applied for the first time a proteomic approach to the rat model of PRS that recapitulates some of the features of stress-related disorders in humans [14,15]. This model is valuable for the study of the pathological epigenetic programming induced by stressful events occurring early in life (see Introduction and references therein). We found that PRS altered the expression profile of several hippocampal proteins, including proteins involved in signal transduction, intracellular trafficking and membrane fusion of synaptic vesicles. Interestingly, some of the proteins modified by PRS such as synapsin 2, LASP 1 and prohibitin, are known to be glucocorticoid regulated. This is relevant because PRS rats present an increased secretion of glucocorticoid in response to stress [16]. Indeed, inactivation of glucocorticoid receptor in the hippocampus reduces levels of synapsins in mice [26], and acute corticosterone treatment enhances Lim family proteins [27], among which there is LASP1 , a dynamic focal adhesion protein involved in mechanisms of cell migration and survival [28,29]. Prohibitin also was modified by PRS. Such protein is a membrane-bound chaperone which inhibits DNA synthesis

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Table 1 – Selected list of proteins whose expression was modified in the hippocampus of PRS rats. Proteins were separated by 2D electrophoresis, and nano-LC–MS/MS analysis was performed after trypsin digestion on silver-stained spots. The biological function of the identified proteins is indicated based on gene ontology. Accession number, entry name, and theoretical MW and pI are indicated, according to the UniProtKB database on the Expasy server. For each candidate, the Mascot score and the number of matched peptides obtained from the MS/MS ion Search module (Mascot) are indicated. Spot numbers are reported according to Fig. 1A. (*) Synapsin-2 and vesicle-fusing ATPase proteins have been identified in spot nos. 39 and 50 (see Suppl Table 1). The higher score obtained for each of these candidates is reported here.

Spot	Biological process	Protein name	Entry name	Accession	MW Kda	pI	Mascot score	Peptides
	Signal transduction							
19		CaMK2	KCC2A	P11275	54	6.6	62	1
13		Phytanoyl CoA hydroxylase interacting protein	PHYYIP	Q568Z9	38	6.5	134	2
	Synaptic vesicles							
39		Synapsin-2(*)	SYN2	Q63537	63	8.73	78	1
26		Synaptosomal-associated protein 25	SNAP25	P60881	23	4.66	235	8
3		Syntaxin binding protein 1	STXB1	P61765	68	6.49	161	4
	Protein synthesis							
9		Prohibitin	PHB	P67779	29	5.5	191	5
50		Elongation factor 1-alpha 1	EF1A1	P62630	50	9.10	67	1
19		T-complex protein1 beta subunit	TCPB	Q5XIM9	57	6.01	89	2
19		F-box/LRR-repeat protein 16	FXL16	Q5MJ12	52	6.1	147	3
	Cytoskeleton dynamics							
19		Fascin	FSCN_1	P85845	55	5.8	169	5
13		LASP-1	LASP1	Q99MZ8	30	6.5	64	2
15		Dihydropyriminidase-like2	DPYL2	P47942	62	5.9	321	8
24		Guanine nucleotide-binding protein G(olf) subunit alpha	GNAL	P38406	45	6.23	39	1
50		Vesicle-fusing ATPase(*)	NSF	Q9QUL6	83	6.55	51	3
17		Mitochondrial import receptor subunit TOM70	TOM70	Q75Q39	68	7.4	117	3
	Energetic metabolism							
3		Transferrin	TRFE	P12346	78	7.14	121	3
10		Phosphomannose isomerase	PMI	Q68FX1	47	5.7	187	4
1		6-phosphofructokinase type C	K6PP	P47860	86	6.95	175	5
10		Adenosine kinase	ADK	Q64640	40	5.7	66	2
29		ATP synthase subunit gamma, mitochondrial	ATPG	P35435	30	8.87	37	2
23		Glucose-6-phosphate 1-dehydrogenase	G6PD	P05370	60	5.97	64	3
24		Isocitrate dehydrogenase [NAD] subunit alpha	IDH3A	Q99NA5	40	6.47	247	7
29		Nitrilase homolog 2	NIT2	Q497B0	31	6.9	41	2
20		Pyruvate kinase isozymes M1/M2	KPYM	P11980	58	6.63	521	18
19		Succinate-semialdehyde-deydrogenase	SSDH	P51650	56	8.3	97	2
19		Tryptophanyl-tRNA-ligase	SYWC	Q6P7B0	54	6.0	145	2

and has been implicated in aging, mitochondrial inheritance and apoptosis (for review see Ref. 30). The increment of prohibitin induced by PRS is in line with other reports about increased probitin levels after glucocorticoids exposure during early postnatal life such as maternal separation [31] or chronic stress (restraint) in adult life [32]. Thus, we provide the first evidence that *in utero* exposure to stress persistently affects the expression in the hippocampus of proteins from different functional categories, which are known to be regulated by stress and/or glucocorticoids. This observation underlines the putative involvement of the early exposure to glucocorticoids in the permanent modification of the hippocampal proteome in the PRS model.

PRS also increased expression of Fascin, an actin-bundling protein that lies downstream of the GTP-binding protein, Rab35, in the regulation of cytoskeleton dynamics and formation of filopodia and growth cones [33,34]. The fascin-encoding gene, FSCN1, is positively regulated by CREB and is induced during neuronal differentiation of NT2 precursor cells [35]. In addition, fascin is up-regulated in neuroectodermal spheres derived from human embryonic stem cells, and is highly expressed in the subventricular zone of the fetal mouse brain [36]. These data suggest that fascin coordinates cytoskeletal changes associated with neuronal differentiation, although the precise role of this protein in the adult hippocampal neurogenesis remains to be determined. PRS rats showed an increased expression of fascin in spite of the observed reduction of phospho-CREB levels and neurogenesis in the hippocampus [18,20,23]. In contrast, fascin is down-regulated in the ventral hippocampus of normal rats treated with the antidepressant, escitalopram [30]. Perhaps fascin acts as a negative regulator of adult neurogenesis and antidepressants enhance neurogenesis by reducing the expression of fascin. This interesting hypothesis warrants further investigation.

A number of proteins involved in cellular metabolism were modified by PRS. One example was phosphomannose isomerase, a key enzyme in the biosynthetic pathway of N-glycosylprotein [37]. Protein glycosylation critically regulates different aspects of neuronal function including synaptic plasticity [38], and has been implicated in the pathophysiology of neurodegenerative disorders [39,40]. However, inactivating mutations of phosphomannose isomerase causes the congenital disorder

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of glycosylation type Ib, in which the CNS is not affected [41,42]. Thus, the precise relationship between phosphomannose isomerase and the pathological phenotype of PRS rats remains to be determined. Other identified proteins were 6-phosphofructokinase, pyruvate kinase and glucose-6-phosphate dehydrogenase, three enzymes involved in glucose utilization and energetic metabolism that are activated by insulin [43,44]. Changes in the expression of the three enzymes could contribute to the development of insulin resistance and altered glucose metabolism seen in PRS rats [45,46]. A decreased energetic metabolism is expected in light of the depressive phenotype and the negative resilience to stress exhibited by PRS rats [14,15,47].

In conclusion, our data offer the first evidence that PRS induces long-lasting changes in the expression profile of hippocampal proteins that likely reflect a pathological epigenetic program triggered in the perinatal life. Anxiety generated by restraint stress in pregnant mothers [48] might influence brain development during the fetal life as a result of malnutrition or excessive exposure to maternal corticosteroids [49]. Alternatively, the epigenetic misprogramming of PRS rats can be the consequence of the low maternal care in the first week of postnatal life induced by gestational stress (personal observations from the laboratory). The latter hypothesis is more likely because there is compelling evidence that low maternal care causes permanent changes in gene function and behavior in the offspring [2,7,50], and cross-fostering, which increased maternal care, prevents at least the abnormal HPA response to stress induced by PRS in particular on MR and GR hippocampal receptors [16]. Changes in hippocampal proteins seen in PRS rats may facilitate the identification of novel molecular processes and candidate genes involved in the regulation of the stress response and in the pathophysiology of mood disorders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2011.12.017.

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2. Anxiety-like behavior in the prenatally stressed rat is associated with a selective reduction of glutamate release in the ventral hippocampus.

Preface

In the previous section we found that PRS affected the proteomic profile in the hippocampus of adult rats. As mentioned above, the majority of neuroproteomic studies have been conducted on hippocampus in animal models of depression (Martins-de-Souza *et al.*, 2010). Indeed, ventral hippocampus seems a key brain region to investigate the neuroproteomic of stress-related disorders (Bisgaard *et al.*, 2007; Marais *et al.*, 2009). In particular, proteomic approaches have produced a draft profile of the overall molecular composition of the mammalian neuronal synapse. It appears that synapses have over 1000 protein components and the mapping of their interactions, organization and functions will lead to a global view of the role of synapses in physiology and disease (Grant *et al.*, 2005). A major functional subcomponent of the synaptic machinery is a multiprotein complex of glutamate receptors and adhesion proteins with associated adaptor and signaling enzymes, totally 185 proteins known as the NMDA receptor complex/MAGUK associated signaling complex (NRC/MASC). Also, the postsynaptic density protein complex has long been a major target of proteomics in neuroscience, as the number of glutamate receptors on a synapse is one of the main determinants of synaptic efficacy (Shinohara, 2012). In our proteomic study, we have also shown that more than 20% of PRS-affected proteins were associated to neural plasticity and synaptic transmission. Regulated neurotransmitter release depends on Ca^{2+} sensors, C2 domain proteins that associate with phospholipids, the three proteins of the SNARE complex (VAMP, SNAP25, syntaxin), and other proteins regulating the trafficking of synaptic vesicles, such as synaptophysin, synapsins, munc-18, and Rab3A (Han *et al.*, 2010; Epp *et al.*, 2011; Hussain and Davanger, 2011; Walter *et al.*, 2011) (Box1). Different synapses have the ability to release multiple vesicles in response to a single action potential (Tong and Jahr, 1994). Multivesicular release is dynamically regulated by activity and auto-feedback signals (Oertner *et al.*, 2002; Wadiche and Jahr, 2001). For example, presynaptic $GABA_B$ receptors can suppress multivesicular release to decrease the synaptic glutamate concentration (Chalifoux and Carter, 2011). Also, the blocking of mGlu2/3 receptors negatively regulates glutamate release in the brain (Nicoletti *et al.*, 2011). Increasing evidence suggests that impairment of glutamate levels in specific brain regions were associated to stress-related disorders (Popoli *et al.*, 2012).

In this section we analyzed the glutamatergic synapse in PRS rats, by measuring both glutamate release in hippocampal superfused synaptosomes (for further detail on release method, see Box2) and expression of key proteins of the synaptic maintenance in

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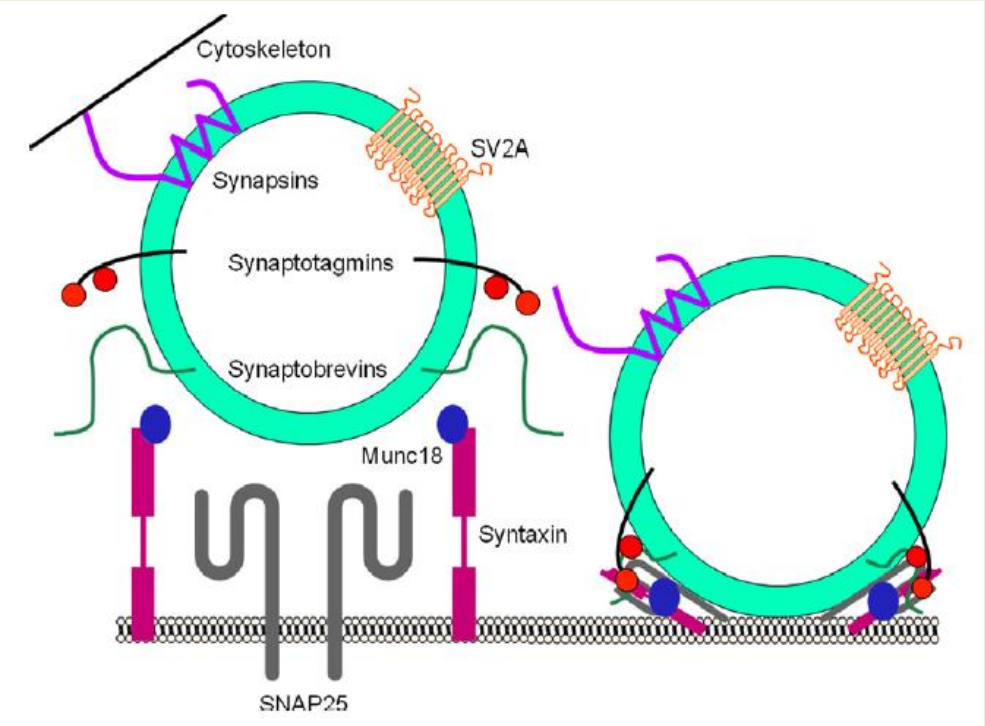
hippocampus. The aim of this work was to assess the role of the hippocampal glutamatergic system in the programming of the anxious/depressive phenotype of PRS rats. Since PRS showed a reduced glutamate release in the ventral hippocampus, we used a cocktail of drugs that block presynaptic mGlu2/3 receptors and $GABA_B$ receptors – that we demonstrate, for the first time, to enhance glutamate release - to examine whether the modulation of glutamate release in hippocampus could be related to anxiety-like behavior.

BOX 1:

Schematic representation of the proteins involved

in the docking of synaptic vesicles.



Synapsins and SV2A allow regulation of the readily releasable pool of vesicles. Synaptobrevins (VAMP), SNAP-25, and syntaxin form the SNARE complex, which binds directly with Munc-18 to allow fusion with the membrane and release of neurotransmitter (from Rowley *et al.*, 2012).

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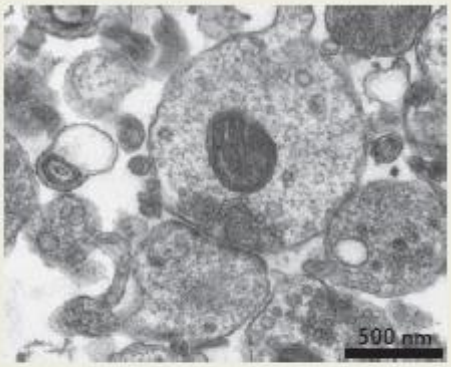
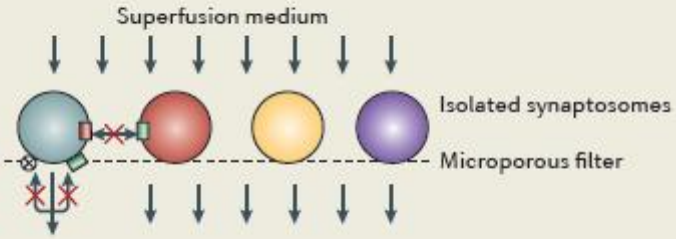
BOX 2:

Release of endogenous neurotransmitters from purified synaptosomes

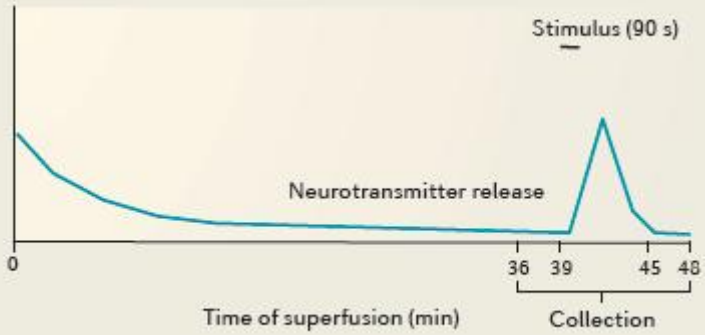
A



B



C



Release of neurotransmitters elicits a chain of reaction that results in the change of the release of neurotransmitter (glutamate in our example). This may confound data on synaptic release in nerve terminals. Instead, this technique, originally developed by Raiteri and co-workers at the University of Genoa (Raiteri *et al.*, 1974), consist in stratifying a thin layer of (semi)purified synaptosomes (A) on a microporus filter and applying a constant up-down superfusion to the sample (B). Through this method, any released endogenous neurotransmitter and modulators are immediately removed by the superfusion method, thereby preventing both the re-uptake by transporters and the activation of autoreceptors or heteroreceptors on synaptic terminals. Thus, indirect effects on glutamate release are strongly minimized or prevented. For further details on protocol and neurotransmitter collection (C), please see Method section.
(adapted from Popoli *et al.*, 2012)

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Behavioral/Systems/Cognitive

Anxiety-Like Behavior of Prenatally Stressed Rats Is Associated with a Selective Reduction of Glutamate Release in the Ventral Hippocampus

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Abnormalities of synaptic transmission and plasticity in the hippocampus represent an integral part of the altered programming triggered by early life stress. Prenatally restraint stressed (PRS) rats develop long-lasting biochemical and behavioral changes, which are the expression of an anxious/depressive-like phenotype. We report here that PRS rats showed a selective impairment of depolarization- or kainate-stimulated glutamate and [^{3}H]D-aspartate release in the ventral hippocampus, a region encoding memories related to stress and emotions. GABA release was unaffected in PRS rats. As a consequence of reduced glutamate release, PRS rats were also highly resistant to kainate-induced seizures. Abnormalities of glutamate release were associated with large reductions in the levels of synaptic vesicle-related proteins, such as VAMP (synaptobrevin), syntaxin-1, synaptophysin, synapsin Ia/b and IIa, munc-18, and Rab3A in the ventral hippocampus of PRS rats. Anxiety-like behavior in male PRS (and control) rats was inversely related to the extent of depolarization-evoked glutamate release in the ventral hippocampus. A causal relationship between anxiety-like behavior and reduction in glutamate release was demonstrated using a mixture of the mGlu2/3 receptor antagonist, LY341495, and the $GABA_B$ receptor antagonist, CGP52432, which was shown to amplify depolarization-evoked [^{3}H]D-aspartate release in the ventral hippocampus. Bilateral microinfusion of CGP52432 *plus* LY341495 in the ventral hippocampus abolished anxiety-like behavior in PRS rats. These findings indicate that an impairment of glutamate release in the ventral hippocampus is a key component of the neuroplastic program induced by PRS, and that strategies aimed at enhancing glutamate release in the ventral hippocampus correct the "anxious phenotype" caused by early life stress.

Introduction

The effects of stress on the brain have long been associated with the onset and exacerbation of several neuropsychiatric disorders such as depression, anxiety, drug addiction, and epilepsy (McEwen, 2012). Alterations in glutamate neurotransmission are believed to play a role in the pathophysiology of such disorders (Ongür, 2008; Chen et al., 2010). Exposure to stress and treatment with glucocorticoids alter glutamatergic neurotransmission and neuroplasticity in brain regions related to depression and anxiety, such as the hippocampus, amygdala, and prefrontal cortex (Mozhui et al., 2010; reviewed by Popoli et al., 2012). Musazzi et al. (2010) have shown that acute stress led to an accumulation of presynaptic SNARE complexes in cortical synaptic membranes, thus raising the interesting possibility that stress directly affects the presynaptic machinery of glutamate release. The study of glutamate release in response to chronic stress is still at its infancy (Moghaddam, 2002; Yamamoto and Reagan, 2006), and there are no data on how early life stress affects glutamate release in the adult life. The latter issue is particularly relevant because early life stress causes long-lasting changes in neuroplasticity that result in an increased vulnerability to stress-related disorders in adult life (Meaney et al., 2007; Darnaudéry and Maccari, 2008; Lupien et al., 2009). Prenatal restraint stressed (PRS) rats represent a model that recapitulates some of the features of depression and anxiety (Maccari et al., 1995; Dugovic et al., 1999; Darnaudéry et al., 2006; Maccari and Morley-Fletcher, 2007; Zuena et al., 2008; Van Waes et al., 2009; Laloux et al., 2012; Mairesse et al., 2012a). Interestingly, male PRS rats show a prominent anxious-like phenotype, whereas female PRS rats are more prone to develop a depressive-like phenotype (Zuena et al., 2008; Morley-Fletcher et al., 2011; Van Waes et al., 2011). PRS rats show also a

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reduced number of proteins involved in signal transduction and neuroplasticity regulation as revealed by a recent mass spectrometry analysis (Mairesse et al., 2012b). Most of these changes are reversed by chronic antidepressant treatment (Morley-Fletcher et al., 2003, 2004, 2011; Mairesse et al., 2012a). Hence, PRS rats represent an animal model of stress-related disorders that meets the criterion of construct validity because it replicates environmental factors implicated in the etiology of depression and anxiety (Krishnan and Nestler, 2008, 2010). In addition, most of the abnormalities in synaptic transmission and plasticity in the hippocampus of PRS rats are seen in the ventral hippocampus (Zuena et al., 2008; Morley-Fletcher et al., 2011), the specific portion of the hippocampus that encodes memories related to stress and emotions (Fanselow and Dong, 2010). We report here that male PRS rats show a selective impairment of glutamate release in the ventral hippocampus associated with anxiety and a reduced expression of the SNARE proteins and vesicle-associated proteins, as well as the mammalian uncoordinated-18 Munc-18, and the glutamate terminal-specific monomeric GTP-binding protein, Rab3a. Pharmacological enhancement of glutamate release in the ventral hippocampus corrected the anxious-like phenotype of PRS rats.

Materials and Methods

Animals

Forty nulliparous female Sprague Dawley rats (20 for control and 20 for PRS groups), weighing ~250 g, were purchased from a commercial breeder (Charles River). Animals were housed at constant temperature (22 ± 2°C) and under a regular 12 h light/dark cycle (lights on at 8.00 A.M.). Pregnant females were randomly assigned to stressed or control groups. ($n = 12$ per group).

Stress protocol

Animals were subjected to PRS according to our standard protocol (Maccari et al., 1995; Morley-Fletcher et al., 2003). From day 11 of pregnancy until delivery, pregnant female rats were subjected to three stress sessions daily (45 min each), during which they were placed in transparent plastic cylinders and exposed to bright light. Only male offspring from litters containing 10–14 pups with a comparable number of males and females were used for the experiments. A maximum of one or two male pups were taken from each litter for each measure to remove any litter effects (Becker and Kowall, 1977; Chapman and Stern, 1979). All experiments followed the rules of the European Communities Council Directive 86/609/EEC. The local ethical committee approved the prenatal stress procedure. We used the same sets of animals (3 month olds) for anxiety and glutamate release (see correlation); in microinfusion experiments we used the same sets of animals (3 month olds) for the two tests of anxiety. For the other experiments we used separate sets of animals (2 month olds).

Assessment of glutamate and GABA release in superfused synaptosomal preparations

Purified synaptosomes isolated from the ventral and the dorsal hippocampus (dissected as described by Robertson et al., 2005), the perirhinal cortex, the prefrontal cortex, the amygdala, and the striatum were prepared as described by Dunkley et al. (1986), with minor modifications. Briefly, the tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with TRIS (final concentration 0.01 M) using a glass Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged at 1000 × g for 5 min, to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll gradient (6, 10, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 × g for 5 min. The layer between 10 and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. The synaptosomal pellet was then resuspended in physiological medium (standard medium) with the following composition (in mM): 140 NaCl, 3 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 1.2 NaH_2PO_4, 5 $NaHCO_3$, 10 mM HEPES, and 10 glucose, pH 7.2–7.4. Synaptosomal protein levels were determined according to Bradford (1976). Synaptosomes were incubated for 15 min a 37°C in a rotary water bath in the absence (experiments of endogenous glutamate and GABA release) or presence of [2,3-^{3}H]D-aspartate (20–50 nM; sp. act. 11.3 Ci/mmol, PerkinElmer).

Identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel chambers in a Superfusion System (Raiteri and Raiteri 2000; Ugo Basile) maintained at 37°C and superfused at 0.5 ml/min with standard physiological solution.

When studying the release of neurotransmitter evoked by kainic acid (Tocris Bioscience) or depolarizing concentrations of K^+, synaptosomes were transiently (90 s) exposed, at $t = 39$ min, to 10 μM kainic acid or to 20 (amygdala) or 12 (all other regions) mM K^+ (substituted for NaCl in the superfusate). Superfusion was always performed with media containing 50 μM amino-oxyacetic acid (Sigma) to inhibit GABA metabolism. Three superfusate fractions were collected according to the following scheme: two 3 min fractions (basal release), one before ($t = 36–39$ min, b1) and one after ($t = 45–48$ min, b3) a 6 min fraction ($t = 39–45$ min; evoked release, b2). Fractions collected and superfused synaptosomes were counted for radioactivity or for endogenous amino acid content. Endogenous glutamate and GABA were measured by HPLC analysis after precolumn derivatization with o-phthalaldehyde and separation on a C_{18} reverse-phase chromatographic column (10 × 4.6 mm, 3 μm; at 30°C; Chrompack) coupled to a fluorimetric detector (excitation wavelength, 350 nm; emission wavelength, 450 nm). Buffers and the gradient program were as follows: solvent A, 0.1 M sodium acetate, pH 5.8/methanol, 80:20; solvent B, 0.1 M sodium acetate, pH 5.8/methanol, 20:80; solvent C, 0.1 M sodium acetate, pH 6.0/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; isocratic flow, 2 min; 78% A and 22% B in 2 min; isocratic flow, 6 min; 66% A and 34% B in 3 min; 42% A and 58% B in 1 min; 100% B in 1 min; isocratic flow, 2 min; 100% C in 3 min; flow rate, 0.9 ml min^{-1}. Homoserine was used as the internal standard. Synaptosomal protein contents were determined according to Bradford (1976). The amount of endogenous glutamate and GABA from synaptosomes in superfusate fractions was expressed as picomoles per milligram of protein (pmol mg^{-1} protein). Radioactivity in each superfusate fraction was quantified by liquid scintillation. The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). The depolarization-induced overflow was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6 min fraction collected during and after the depolarization pulse (evoked release, b2).

Assessment of [^{3}H]D-aspartate release in hippocampal slice preparations

Slices (0.4 mm thick) from the dorsal or ventral hippocampus were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering) and then placed in a superfusion medium with the following composition (in mM): 125 NaCl, 3 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 1 NaH_2PO_4, 22 $NaHCO_3$, and 10 glucose (aeration with 95% O_2 and 5% CO_2), pH 7.2–7.4, at 2–4°C. Slices were rinsed by changing the physiological solution every 20 min. Slices were labeled with 90 nM [^{3}H]D-aspartate (20 min at 37°C) in standard medium in an atmosphere of 95% O_2 and 5% CO_2. After washing with tracer-free medium, slices were transferred to parallel superfusion chambers (one slice/chamber) and superfused (1 ml/min at 37°C). After 60 min of superfusion to equilibrate the system, six 5 min samples were collected. Slices were exposed to 30 mM K^+ in the absence or presence of 3-[[(3,4-dichlorophenyl)methyl]amino]propyl] diethoxymethyl) phosphinic acid (CGP52432; Tocris Bioscience) and (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495; Tocris Bioscience) for 5 min, starting from $t = 70$ min of superfusion. Drugs were added from $t = 30$ min of superfusion until the end of the experiments. Samples collected and solubilized slices (Soluene; Canberra Packard) were counted for radioactivity. The amount of radioactivity released into each superfusate fraction was expressed as fractional efflux (see above). Drug effects were expressed as "induced overflow" and were

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estimated by subtracting the neurotransmitter content into the second and the fifth fractions collected from that in the third and in the fourth fractions collected.

In vivo studies

Kainate-induced motor seizures and electroencephalography/electromyography recording

We assessed kainate-induced limbic motor seizures in separate groups of control and PRS rats ($n = 6$ per group). Kainate-induced seizures represent an established experimental animal model of temporal lobe epilepsy in humans (Sharma et al., 2007; Joëls, 2009). Animals underwent 2 weeks of habituation to electroencephalogram (EEG)/electromyogram (EMG) recording before behavioral assessment for motor seizures. Rats were anesthetized with ketamine/xylazine (75/10 mg/kg, i.m.). Electrodes for EEG recordings were chronically implanted using a stereotaxic apparatus. Three stainless steel screw electrodes were threaded through the skull bilaterally over the frontal and parietal cortex to record the EEG. One electrode threaded through the midline of the frontal bone was used as ground. Teflon-coated multistranded stainless steel wires with 2 mm exposed at the tips (Goodfellow Sarl) were placed in the dorsal neck muscles to record the EMG. EEG and EMG leads were attached to a connector (MS363; Plastics One) and fixed to the skull with dental acrylic.

Recording. For registration, the electrodes were connected to a preamplifier (8213; Pinnacle Technology) through the plastic connector. This preamplifier avoids the registration of electrical interferences. The preamplifier was connected to a rotating swivel allowing free animal movements, and the swivel was connected to the EEG/EMG Data Conditioning and Acquisition System (8206; Pinnacle Technology), which was USB linked to a computer. Signal acquisition was performed using the Sirena acquisition suite (Pinnacle Technology). The EEG and EMG were recorded at a frequency of 400 Hz. Both EEGs were lowpass filtered at 40 Hz. EMG signals were highpass filtered at 10 Hz and subjected to a 100 Hz lowpass cutoff. After surgery, rats were individually housed in Plexiglas cages (30 cm diameter, 40 cm high), and left undisturbed for a postsurgery recovery period of 2 weeks. During the second week of recovery, rats were habituated to the EEGs/EMG recording procedure for the following 2 weeks. Habituation consisted of two recording sessions of 8 h and one session of 24 h. At the end of the habituation period, the day of the experiment, EEGs/EMG recordings started 1 h after the light switch-on and continued for the next 8 h. Two hours after the beginning of the registration, kainate (Tocris Bioscience) was injected intraperitoneally at doses (7 mg/kg) in the same range as those reported by previous studies (Sperk et al., 1983; Berg et al., 1993), and were proven to cause full limbic motor seizures in control rats. The presence of characteristic spikes and/or spike clusters activity was correlated to each stage of behavioral seizure, after kainate injection.

Kainate-induced seizures. Motor seizures were observed for 4 h following systemic kainate injection, and manually scored according to Racine (1972), as follows: 0, absence of seizures; 1, staring spells, immobilization, and hypoactivity; 2, paroxysmal wet dog shake and head nodding; 3, motor seizures associated with masticatory movements and tail arching; 4, rearing with forelimb jerks and salivation; 5, generalized convulsions with loss of postural control and intense myoclonic jerks lasting at least 1 h; and 6, "full status epilepticus" and death.

Assessment of anxiety-like behavior

We assessed anxiety-like behavior in control and PRS rats by using the elevated plus maze (EPM) and the light–dark tests. All animals used for *ex vivo* measurements of neurotransmitter release and immunoblot analysis of protein expression had been tested for anxiety-like behavior at least 1 week earlier. The EPM test was performed essentially as described by Pellow et al., 1985. The test was performed between 13:00 and 16:00 h, lasted for 5 min, and began with the placement of the rat in the center of the maze with the head facing a closed arm. The time spent in open and closed arms was recorded on-line by a video camera and the percentage of time spent in open arms was calculated. We also measured the number of entries into the open and closed arms, the number of crossings through the center, the number of episodes of head dips over the size of the maze, the number of episodes of rearing, and the latency to enter the open and the closed arms.

The light and dark box setup consisted of two compartments: one light compartment (45 × 32 × 32 cm, 50 lux; light box) and one dark compartment (30 × 32 × 32 cm, 5 lux). The compartments were connected via a small opening (10 × 15 cm) enabling transition between the two boxes. Rats were placed in the light compartment and the time spent in each compartment and the latency to the first entry into the light compartment during the 5 min test, were assessed on-line via a video camera located above the box. Behavior was automatically analyzed using video tracking software (View Point).

Microinfusions of CGP52432 and LY341495 in the ventral hippocampus

All control and PRS rats used for these experiments had been tested for anxiety at the light–dark box 1 week before surgery. Rats were injected intraperitoneally with an anesthetic solution containing ketamine (100 mg/kg), xylazine (8 mg/kg), and acepromazine (1 mg/kg), placed into a David Kopf stereotaxic apparatus with the incisor bar 5.0 mm above the interaural line, and bilaterally implanted with permanent cranial guide cannulae (22 gauge; Plastic One) into the ventral hippocampus (anteroposterior + 5.5; mediolateral ± 4.5; dorsoventral −5.5 mm from bregma and skull; Paxinos and Watson, 2007). Cannulae were fixed with dental acrylic cement directly anchored to the skull. After surgery, obturators were inserted into the guide cannulae, rats were returned to their home cage and were left undisturbed for a 7–10 d recovery period. Twelve control and 12 PRS rats were selected for microinfusion experiments and behavioral analysis. LY341495 and CGP52432 were dissolved in PBS (1.05 mM KH_2PO_4, 2 Na_2HPO_4, 3 mM H_2O, 154 mM NaCl, pH 7.4) to obtain final concentrations of 100 pg/μl LY341495 and 1 ng/μl CGP52432. After 2 d of habituation to microinjection procedures, two groups of control and two groups of PRS rats received bilateral injections of either PBS alone (vehicle) or PBS containing CGP52432 and LY341495. The internal injection cannulae were connected to lengths of polyethylene tubing that in turn were connected to 10.0 μl Hamilton syringes. Injections were made bilaterally in a volume of 1 μl/side over a period of 2 min. After 1 min, the injection cannulae were withdrawn, the obturators replaced, and rats were returned to their home cage for 15 min before the start of behavioral assessments. All rats underwent two consecutive tests of 5 min in the light–dark box and the EPM, as described above. The two tests were performed one immediately after the other. This behavioral protocol may confound data of the second test (the EPM) because the stress associated with the execution of the first test (the light–dark box) might differentially affect the performance in the EPM in the four group of rats (control rats injected with vehicle, PRS rats injected with vehicle, control rats injected with CGP52432 plus LY341495, and PRS rats injected with CGP52432 plus LY341495. Despite these potential limitations, the execution of two consecutive tests was necessary to avoid the bias of reinjecting the mixture of drugs in the same animals without having knowledge of the neuroadaptive changes that intrahippocampal injection of CGP52432 and LY341495 may cause.

The correct position of the guide cannula in the ventral hippocampus was confirmed in all rats by injection of 1 μl of methylene blue (5%, dissolved in saline).

Western blot analysis

Two groups of control and PRS rats ($n = 6$ per group) were killed by decapitation and the ventral and dorsal portions of the hippocampus were rapidly dissected (Robertson et al., 2005). To isolate synaptosomes, tissue was manually homogenized with a potter in 10 vol of HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES, pH 7.4). All procedures were performed at 4°C. Homogenates were centrifuged at 1000 × *g* for 10 min and resulting supernatants were centrifuged at 10,000 × *g* for 15 min. The pellet obtained from the second centrifugation was resuspended in 10 vol of HEPES-buffered sucrose and then spun again at 10,000 × *g* for 15 min. This pellet contained the crude synaptosomal fraction. To validate the purity of this synaptosomal fraction we used anti-histon H3, anti-β-tubulin, anti-synapsin Ia/b in immunoblot analysis. BCA assay was used to determine protein concentration. Synaptosomes lysates

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were resuspended in Laemmli reducing buffer and 20 μg of each sample were first separated by electrophoresis on Criterion TGX 4–15% precast SDS-polyacrylamide gels (Bio-Rad) and later transferred to nitrocellulose membranes (Bio-Rad). Transfer was performed at 4°C in a buffer containing 35 mM TRIS, 192 mM glycine, and 20% methanol. We used the following primary antibodies: rabbit polyclonal anti-synapsin Ia/b (sc-20780, 1:4000), rabbit polyclonal anti-synapsin IIa (sc-25538, 1:4000), rabbit polyclonal anti-synaptophysin (sc-9116, 1:80,000), rabbit polyclonal anti-VAMP (synaptobrevin, sc-13992, 1:2000), rabbit polyclonal anti-syntaxin-1 (sc-13994, 1:5000), and mouse monoclonal anti-SNAP-25 (sc-136267, 1:10000) (all purchased from Santa Cruz Biotechnology); mouse monoclonal anti-rab3a (107111, 1:2000), mouse monoclonal anti-Munc-18 (116011, 1:2000), mouse monoclonal anti-VGLUT-1 (135511, 1:2000), rabbit polyclonal anti-GluK3 (180203, 1:1000), and rabbit polyclonal GluK5 (180103, 1:1000) (all purchased from Synaptic System); rabbit polyclonal anti-Gluk1 (AGC-008, 1:1000) and rabbit polyclonal anti-GluK2 (AGC-009, 1:1000) (both purchased from Alomone Labs); rabbit polyclonal anti-GluK4 (ab67404, 1:1000) (purchased from Abcam); rabbit polyclonal anti-GLAST (GLAST11-A, 1:1000), rabbit polyclonal anti-GLT-1 (GLT11-A, 1:1000), and rabbit polyclonal anti-EAAC-1 (EAAC11-A, 1:1000) (all purchased from Alpha Diagnostic International); and mouse monoclonal anti-β-actin (A5316, 1:80,000) (purchased from Sigma). Secondary anti-mouse or anti-rabbit antibodies (purchased from GE Healthcare) were used a dilution at 1:10,000.

Densitometric analysis was performed with Quantity One software (Bio-Rad) associated to a GS-800 scanner. The ratio of individual proteins to β-actin was then determined and these values were compared for statistical significance.

Statistical analysis

Data of release experiments, immunoblot analysis, and behavioral data with light–dark box and EPM (excluding data obtained in microinfused animals) were analyzed by Student's *t* test (PRS vs control rats). Data of kainate-induced seizures were analyzed by two-way ANOVA for repeated measures followed by the Neumann–Keuls *post hoc*. Behavioral data obtained after microinfusions with vehicle or CGP52432 + LY341495 were analyzed by two-way ANOVA (group × treatment) followed by the Neumann–Keuls *post hoc*. A p value <0.05 was considered as statistically significant.

Results

PRS selectively reduced depolarization-evoked release of glutamate in superfused synaptosomes isolated from the ventral hippocampus

To study the effects of PRS on neurotransmitter release we used superfused synaptosomes prepared from adult male PRS rats and their age-matched controls. Our superfusion method eliminates the components in neurotransmitter release mediated by the inverse operation of membrane transporters and the influence of endogenous ligands acting at presynaptic receptors, thus allowing a reliable estimation of how the intrinsic release machinery responds to depolarization or other stimuli (Raiteri et al., 1974; Raiteri and Raiteri, 2000). Depolarization-evoked release in this

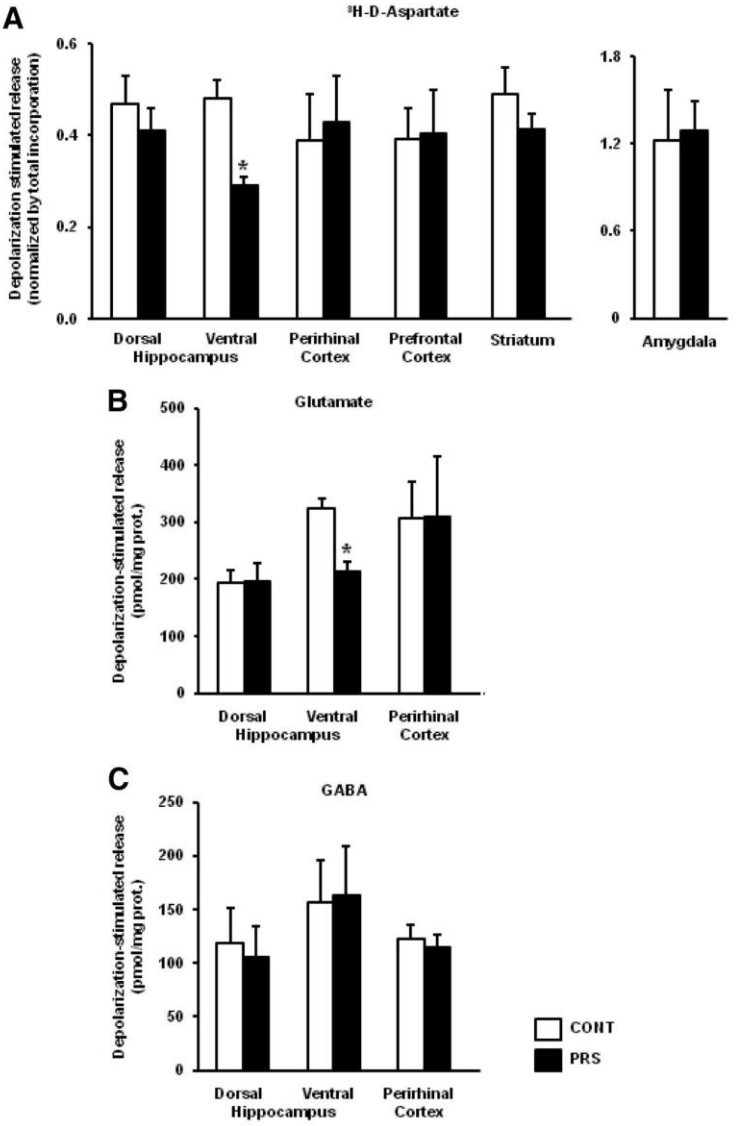

Figure 1. PRS causes a selective impairment of depolarization-evoked glutamate release in synaptosomes from the ventral hippocampus. Superfused synaptosomal preparations from the dorsal and ventral hippocampus, perirhinal cortex, prefrontal cortex, striatum, or amygdala of control (CONT) or PRS rats (one control and one PRS animal in each experiment) were stratified at the bottom of superfusion chambers (three superfusion chambers for each synaptosomal preparation) and superfused as described (see Materials and Methods). The total [^{3}H]D-ASP content in the synaptosomal fraction at the start of the superfusion period amounted, respectively, to control dorsal hippocampus: 221.34 ± 14.75 nCi; PRS dorsal hippocampus: 226.44 ± 13.58 nCi, not significant (n.s.); control ventral hippocampus: 273.21 ± 37.02 nCi; PRS ventral hippocampus: 232.45 ± 21.32 nCi, n.s.; control perirhinal cortex: 341.14 ± 44.81 nCi; PRS perirhinal cortex: 298.31 ± 46.23 nCi, n.s.; control prefrontal cortex: 209.30 ± 18.33 nCi; PRS prefrontal cortex: 218.82 ± 23.44 nCi, n.s.; control striatum: 238.55 ± 15.56 nCi; PRS striatum: 246.07 ± 30.47 nCi, n.s.; control amygdala: 191.13 ± 10.09 nCi; PRS amygdala: 176.89 ± 16.89 nCi, n.s. Data are expressed as nCi/chamber and correspond to the amount of radioactive tracer taken up by each synaptosomal preparation. At $t = 39$ min of superfusion, synaptosomes were challenged with 20 (amygdala) or 12 (all other regions) mM K^+. Synaptosomes were used for measurements of D-[^{3}H]-aspartate (***A***), glutamate (***B***), or GABA (***C***) release. Data are expressed as K^+-induced overflow. Glutamate and GABA overflow is expressed as pmol/mg prot. The evoked release of D-[^{3}H]-aspartate is expressed as the percentage of the total tritium content in synaptosomes. High K^+ depolarization-induced overflow is expressed as stimulated release over basal release. Values are means ± SEM of six experiments run in triplicate (3 superfusion chambers for each experimental condition). *$p < 0.01$ versus the respective controls.

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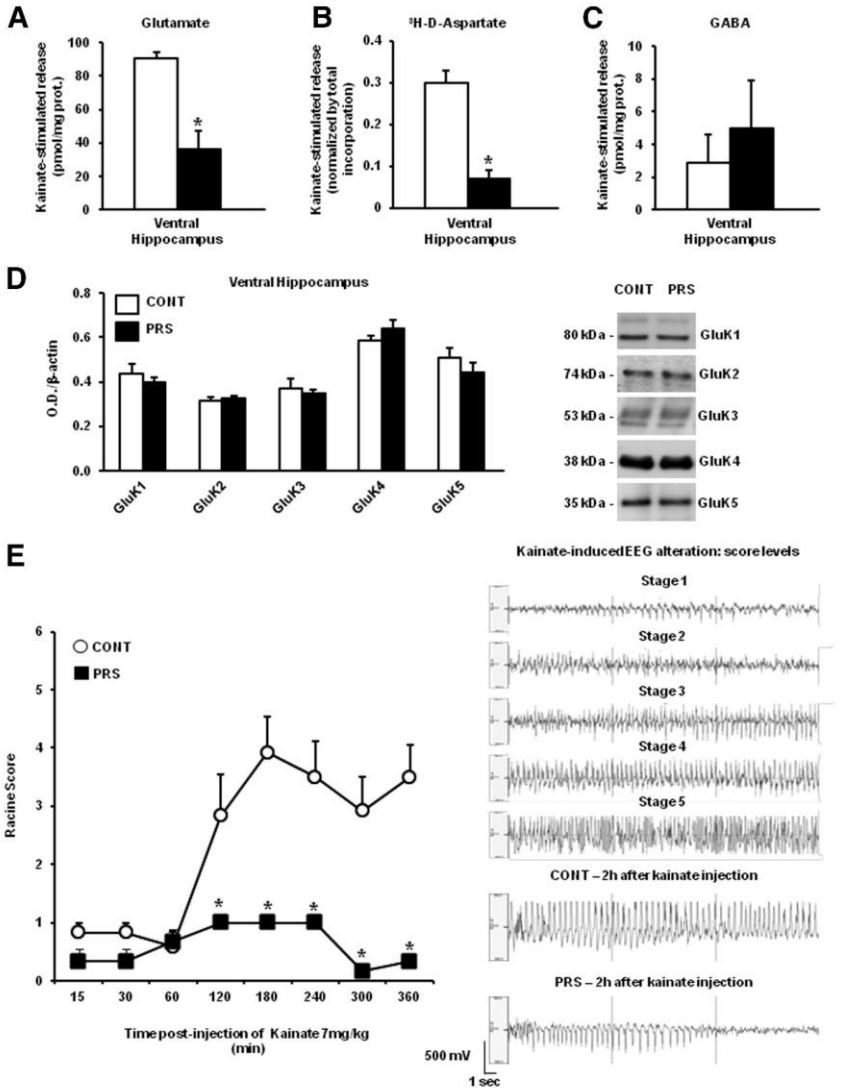

Figure 2. Reduced kainate-stimulated glutamate release in the ventral hippocampus and kainate-induced limbic motor seizures in PRS rats. Kainate-induced release of glutamate, D-[^{3}H]-aspartate, and GABA in superfused synaptosomes prepared from the ventral hippocampus of control (CONT) or PRS rats are shown in ***A***, ***B***, and ***C***, respectively. Data are expressed as reported in Figure 1, as kainate-induced overflow. Values are means ± SEM of six experiments run in triplicate (3 superfusion chambers for each experimental condition). *$p < 0.05$ or $p < 0.01$ versus the respective controls. Immunoblot analysis of GluK1–5 kainate receptor subunits in the ventral hippocampus of control and PRS rats is shown in ***D***. Values are means ± SEM of six determinations. Behavioral score of kainate-induced seizures in control and PRS rats is shown in ***E***. Kainate was injected at the dose of 7 mg/kg, intraperitoneally. Values are means ± SEM of six determinations. *$p < 0.01$ versus the respective data obtained in control rats. Representative EEG traces corresponding to different stages of kainate-induced seizures and representative traces obtained in control and PRS at 2 h following kainate injection.

system is exocytotic and entirely depended on extracellular Ca^{2+} (Bonanno et al., 2005). Synaptosomes prepared from the ventral and dorsal hippocampus, perirhinal cortex, prefrontal cortex, striatum, and amygdala from control or PRS rats were preloaded with D-[^{3}H]-aspartate (a nonmetabolizable analog of glutamate), and then challenged with depolarizing concentrations of K^+. Both uptake and basal (nonevoked) release of D-[^{3}H]-aspartate in all brain regions did not differ between control and PRS rats. In contrast, depolarization-evoked D-[^{3}H]-aspartate release was selectively and substantially reduced in synaptosomes prepared from the ventral hippocampus of PRS rats ($t = 10.70$; $df = 10$; $p < 0.01$). No difference in depolarization-evoked D-[^{3}H]-aspartate release between control and PRS rats was seen in all other brain regions (Fig. 1*A*). In a different set of experiments, synaptosomes from ventral and dorsal hippocampus and perirhinal cortex were challenged with depolarizing concentrations of K^+, and the superfusate was used for measurements of endogenous glutamate and GABA release. Again, depolarization-evoked glutamate release was largely reduced in the ventral hippocampus of PRS rats ($t = 4.70$; $df = 10$; $p < 0.01$), with no changes in the dorsal hippocampus or perirhinal cortex (Fig. 1*B*). Neither basal nor evoked GABA release differed between control and PRS rats in any brain region (Fig. 1*C*).

PRS reduced both kainate-evoked glutamate release in the ventral hippocampus and behavioral responses to kainate

To examine whether the difference between control and PRS rats was stimulus specific, we also challenged synaptosomes with kainic acid. At least in our superfusion system, kainate (10 μM) substantially enhanced both glutamate and GABA release. Again, a substantial reduction in kainate-stimulated glutamate ($t = 2.28$; $df = 10$; $p < 0.05$) and D-[^{3}H]-aspartate ($t = 25.89$; $df = 10$; $p < 0.01$) release was found in the ventral hippocampus of PRS rats (Fig. 2*A*,*B*). In contrast, kainate-stimulated GABA released was unchanged in synaptosomes prepared from the ventral hippocampus of PRS rats (Fig. 2*C*). To exclude that the reduction in kainate-stimulated glutamate release in the ventral hippocampus was due to changes in the expression of kainate receptors, we measured the levels of kainate receptor subunits (GluK1–5) by immunoblotting. GluK1–5 protein levels did not differ between PRS rats and control rats (Fig. 2*D*). As a behavioral counterpart of the study of kainate on glutamate release, we examined kainate-induced motor seizures in control and PRS rats. Systemic injection of kainate (7 mg/kg, i.p.) in control rats induced secondarily generalized partial limbic motor seizures characterized by motor arrest, wet dog shake, head nodding, masticatory movements, and rearing with forepaw clonus. Some control rats developed generalized tonic–clinic seizures and status epilepticus. Interestingly, kainate-induced seizures were largely reduced in PRS rats (group × time $F_{(1,8)} = 11.31$, $p < 0.01$) (Fig. 2*E*). The average seizure severity score of PRS rats at 120–240 min following kainate injection was around "1" of the Racine scale. In contrast, the average score of control rats was between 3 and 4 at 120 min following kainate injection. None of PRS rats showed generalized seizures and status epilepticus in response to kainate (Fig. 2*E*). PRS and control rats did not differ in the temporal latency to the induction of

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motor seizures. Representative EEG traces corresponding to a score of 1 to 5 of the Racine scale are shown in Figure 2*E*. Typical EEG recording of control and PRS rats at 2 h following kainate injection are also shown (Fig. 2*E*).

The reduction of evoked glutamate release was associated with lower expression of synaptic vesicle-related proteins in the ventral hippocampus of PRS rats

We measured the levels of synaptic vesicle-associated proteins and membrane glutamate transporters in purified synaptosomal membranes prepared from the ventral and dorsal hippocampus of control and PRS rats. Substantial reductions in the levels of Rab3A ($t = 4.76$, $df = 10$; $p < 0.01$), Munc-18 ($t = 2.78$; $df = 10$; $p < 0.05$), VAMP (synaptobrevin) ($t = 2.91$; $df = 10$; $p < 0.05$), syntaxin-1 ($t = 3.30$; $df = 10$; $p < 0.01$), synaptophysin ($t = 3.41$; $df = 10$; $p < 0.01$), synapsin Ia/b ($t = 2.65$; $df = 10$; $p < 0.05$), and synapsin IIa ($t = 5.41$; $df = 10$; $p < 0.01$) were found in the ventral hippocampus of PRS rats (Fig. 3*A*), whereas levels of SNAP25, and the glutamate transporters, v-Glut1, GLAST EACC-1, and GLT-1 did not change (Fig. 3*B*). Levels of synapsin Ia/Ib were lowered by as much as 60% and levels of syntaxin by ~50% in the ventral hippocampus of PRS rats. We only found reductions in the levels of synapsin Ia/Ib ($t = 2.70$; $df = 10$; $p < 0.05$) and syntaxin ($t = 3.30$; $df = 10$; $p < 0.01$), and an increase in the levels of v-Glut1 ($t = 2.90$; $df = 10$; $p < 0.05$) in the dorsal hippocampus of PRS rats (Fig. 3*C*,*D*). Levels of all other proteins did not change in the dorsal hippocampus.

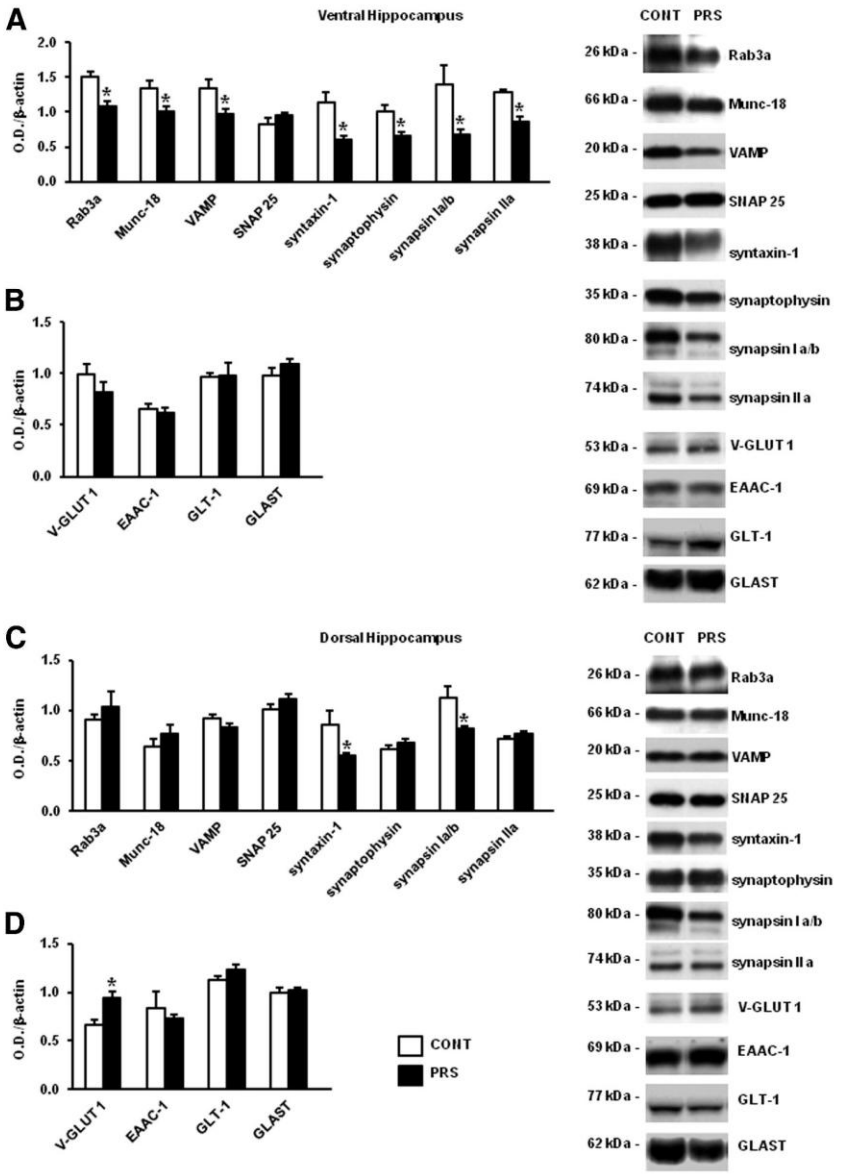

Figure 3. PRS reduced expression of synaptic vesicle-associated proteins in the ventral hippocampus. Immunoblot analysis of SNAREs, vesicle-associated proteins, and glutamate transporters in synaptosomal fractions collected from the ventral (***A***, ***B***) and dorsal (***C***, ***D***) hippocampus of adult PRS and control (CONT) male rats. Values are means ± SEM of six determinations. *$p < 0.05$ or $p < 0.01$ versus the respective controls.

The reduction in depolarization-evoked glutamate release in the ventral hippocampus correlated positively with anxiety-like behavior in PRS rats

PRS rats show anxiety-like behavior (Vallée et al., 1997; Zuena et al., 2008; Morley-Fletcher et al., 2011), and the ventral portion of the hippocampus is involved in emotion and anxiety (Fanselow and Dong, 2010). Hence, we examined the correlation between depolarization-evoked glutamate release in the ventral hippocampus and anxiety-like behavior in control and PRS rats. Both control and PRS rats used for measurements of glutamate release in synaptosomes (see above) had been tested for anxiety-like behavior in the EPM. PRS rats spent less time in the open arm of the EPM, as expected ($t = 3.40$; $df = 10$; $p < 0.01$) (Fig. 4*A*). We found a positive correlation between the time spent by animals in the open arm of the EPM and the extent of depolarization-stimulated glutamate release in the ventral hippocampus ($r = 0.90$; $p < 0.01$), indicating that anxiety-like behavior was inversely related to the evoked release of glutamate (Fig. 4*B*). We extended the study to additional groups of control and PRS rats tested for anxiety-like behavior in the light–dark box. PRS rats spent less time in the light compartment of the light–dark box ($t = 2.90$; $df = 10$; $p < 0.05$) (Fig. 4*C*). There was a positive correlation between the time spent by control and PRS rats in the light compartment and the extent of depolarization-stimulated glutamate release in the ventral hippocampus ($r = 0.89$; $p < 0.01$; Fig. 4*D*), confirming the inverse correlation between anxiety-like behavior and glutamate release.

Pharmacological enhancement of glutamate release in the ventral hippocampus corrects anxiety-like behavior in PRS rats

To examine whether the reduction of glutamate release in the ventral hippocampus was causally related to anxiety-like behavior in PRS rats we used a mixture of drugs that block presynaptic

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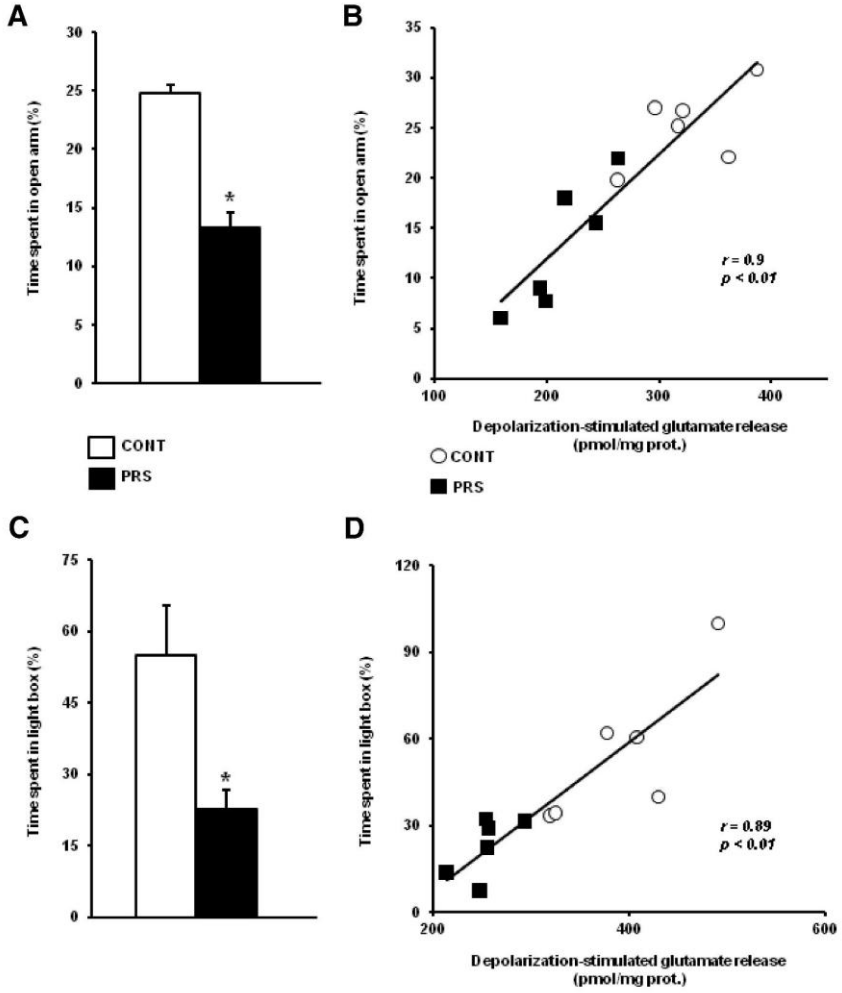

Figure 4. Negative correlation between depolarization-evoked release in the ventral hippocampus and anxiety-like behavior. Anxiety-like behavior in the EPM and light–dark box is shown in ***A*** and ***C***, where the time spent in the open arm of the EPM and in the light compartment of the light–dark box is shown. Different groups of rats were used for behavioral analysis in the EPM and light–dark box. Data are means ± SEM of six determinations. *$p < 0.01$ or $p < 0.05$ versus the respective controls. Correlation analysis between the time spent in the open arm of the EPM or in the light compartment of the light–dark box and depolarization-evoked glutamate release in synaptosomes prepared from the ventral hippocampus of control (CONT) and PRS rats is shown in ***B*** and ***D***, respectively.

type-2/3 metabotropic glutamate (mGlu2/3) receptors and $GABA_B$ receptors. These receptors are known to negatively regulate glutamate release in the hippocampus and other brain regions (reviewed by Chalifoux and Carter, 2011; Nicoletti et al., 2011). We combined the selective $GABA_B$ receptor antagonist, CGP52432 (Lanza et al., 1993) with the preferential mGlu2/3 receptor antagonist, LY341495 (Schoepp et al., 1999). To examine whether this mixture was able to enhance glutamate release we could not use isolated synaptosomal preparations because the method of superfused synaptosomes eliminates the influence of endogenously activated presynaptic receptors on neurotransmitter release (see above). Thus, we measured D-[^{3}H]-aspartate release in preloaded hippocampal slice preparations. We used saturating concentrations of CGP52432 and LY341495 (10 and 1 μM, respectively). At these concentrations, LY341495 is still a preferential blocker of mGlu2/3 receptors with respect to other mGlu receptor subtypes (Schoepp et al., 1999). The addition of CGP52432 and LY341495 enhanced high-K^+ (30 mM) evoked D-[^{3}H]-aspartate release in slices prepared from the ventral hippocampus of both control and PRS rats (39 ± 14 and 29 ± 8% above values obtained with 30 mM K^+ alone, respectively; $n = 5$), without affecting basal D-[^{3}H]-aspartate release. Interestingly, the mixture of CGP52432 and LY341495 had no effect on depolarization-evoked D-[^{3}H]-aspartate release in slices from the dorsal hippocampus of control and PRS rats (data not shown). We therefore decided to study anxiety-like behavior in control and PRS rats after bilateral microinfusion of CGP53432 plus LY341495 in the ventral hippocampus. Based on previous studies (Jackson and Kuehl, 2002; Barker et al., 2006; Li and Pan, 2007;Dong et al., 2012) we first tested three doses of CGP53432 (1 ng, 10 ng, or 50 ng) always combined with 100 pg of LY341495. The mixture containing 10 ng or 50 ng of CGP53432 increased rearing and wet dog shake, whereas the mixture containing 1 ng of CGP53432 did not cause changes in motor activity or spontaneous motor behavior in control rats. Thus, we decided to use 1 ng of CGP53432 combined with 100 pg of LY341495 for the study of anxiety-like behavior in control and PRS rats. All animals used for this study had been tested for anxiety-like behavior in the light–dark box 14–17 d before microinfusion experiments (Fig. 5*A*). Following microinfusion with vehicle or CGP52432 plus LY341495 all animals were consecutively tested in the light–dark box and in the EPM. This behavioral protocol is unusual because data of the second test (the EPM) might have been confounded by the effects of the first test (the light–dark box). However, we adopted this strategy to examine the effect of CGP52432 and LY341495 in two different tests of anxiety without the need to reinject the drugs in the ventral hippocampus. PRS rats infused with vehicle in the ventral hippocampus spent less time in the light compartment of the light–dark box, as expected. This paradigm of anxiety-like behavior was corrected by the mixture of CGP53432 and LY341495. Intrahippocampal infusion of CGP52432 and LY341495 had no effect on control unstressed rats (group × treatment, $F_{(1,20)} = 5.40$; $p < 0.05$; $n = 6$ per group; Fig. 5*B*). PRS rats treated with vehicle showed also an increased latency to enter the light compartment of the box, which, again, was corrected by treatment with CGP53432 and LY341495 (group × treatment, $F_{(1,20)} = 33.54$; $p < 0.01$; Table 1). In contrast, the number of entries into the light and dark compartment did not differ among the four groups of rats (Table 1). The "curative" effect of CGP53432 and LY341495 on anxiety-like behavior of PRS rats was supported by EPM data. PRS rats treated with vehicle, but not PRS rats treated with CGP53432 plus LY341495, spent less time in the open arm of the EPM (group × treatment, $F_{(1,20)} = 18.16$; $p < 0.01$; $n = 6$ per group; Fig. 5*C*). PRS rats treated with vehicle also showed a reduced number of entries into the open arm, a reduced number of episodes of head dips (which

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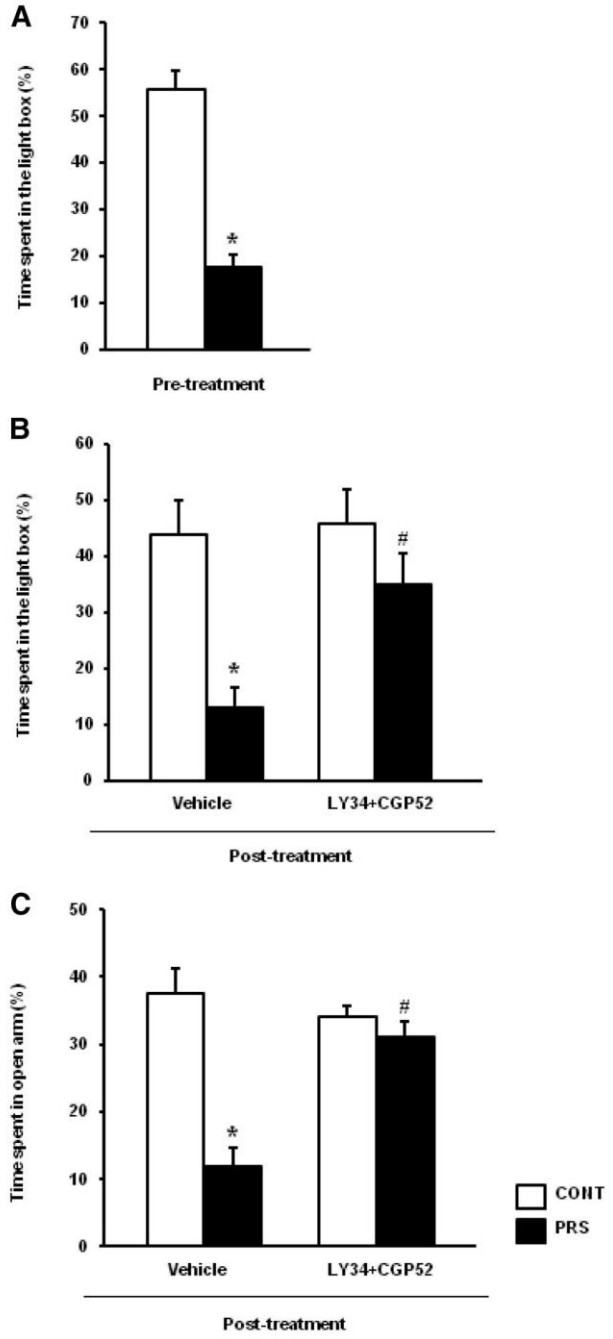

Figure 5. Pharmacological enhancement of glutamate release corrects anxiety-like behavior in PRS rats. All rats were tested in the light–dark box 1 week before surgery (i.e., 14–17 d before drug microinfusions in the ventral hippocampus). The time spent in the light compartment of the light–dark box in this pretest performed 14–17 d before is shown in ***A***. Values are means ± SEM of 12 control (CONT) and 12 PRS rats. *$p < 0.01$ versus control rats. Behavioral data obtained in unstressed and PRS rats following microinfusion of vehicle or CGP52432 plus LY341495 in the ventral hippocampus are shown in ***B*** and ***C***. Animals were first tested in the light–dark box and immediately after in the EPM. The effects of the first test experience might confound the interpretation of data of the second test (the EPM). However, the mixture of CGP52432 and LY341495 reduced anxiety-like behavior in PRS rats in both tests. The time spent in the open arm of the EPM and in the light compartment of the light–dark box in control and PRS rats bilaterally infused with 1 μl PBS containing 1 ng of CGP52432 and 100 pg of LY341495 or PBS alone (vehicle) in the ventral hippocampus are shown in ***B*** and ***C***, respectively. Values are means ± SEM of six rats per group. $p < 0.01$ or $p < 0.05$ versus the respective control values (*) or versus the respective values treated with vehicle (#).

Table 1. Number of entries and latency to enter the two compartments of the light–dark box in control and PRS rats bilaterally infused with vehicle or CGP53432 plus LY341495 into the ventral hippocampus

	Number of entries	Latency to enter (s)
CONT/vehicle		
Light compartment	5.5 ± 0.6	46 ± 9.0
Dark compartment	5.9 ± 0.6	28 ± 9.5
PRS/vehicle		
Light compartment	5.2 ± 1.0	228 ± 31*
Dark compartment	4.7 ± 0.9	9.9 ± 1.8
CONT/ CGP + LY		
Light compartment	5.2 ± 0.9	49 ± 7.3
Dark compartment	5.5 ± 0.8	22 ± 3.2
PRS/CGP + LY		
Light compartment	6.3 ± 0.4	35 ± 3.8
Dark compartment	6.8 ± 0.4	18 ± 3.2

Data were obtained from the same control (CONT) and PRS rats of Figure 5*B*; see legend for details on treatments with CGP53432 and LY341495. Values are means ± SEM of six rats per group. *$p < 0.01$ versus latency to enter the light compartment in all other groups.

is a surrogate indicator for anxiety like-behavior), an increased latency to enter the open arm, and a reduced latency to enter the closed arm. Most of these alterations were corrected by treatment with CGP53432 plus LY341495 (number of entries into the open arm: group × treatment, $F_{(1,20)} = 7.79$; $p < 0.05$; episodes of head dips: $F_{(1,20)} = 25.64$; $p < 0.01$; latency to enter the open arm: $F_{(1,20)} = 5.05$; $p < 0.05$; latency to enter the closed arm: $F_{(1,20)} = 24.20$; $p < 0.05$) (Table 2). The four groups of rats did not differ with respect to the number of entries into the closed arm, the number of crossings through the central area of the EPM, and the episodes of rearing (Table 2), excluding nonspecific effects of the treatments on motor behavior.

Discussion

We have shown for the first time that prenatal restraint stress, which is a model that recapitulates some of the features of depression and anxiety, causes a selective impairment of glutamate release in the ventral hippocampus, a brain region that specifically encodes memories related to stress and emotions (Fanselow and Dong, 2010). The reduced glutamate release in PRS rats was not due to an impaired glutamate synthesis in presynaptic terminals because it was also seen in synaptosomes preloaded with D-[^{3}H]-aspartate. Martisova et al. (2012) found a reduced expression of vesicular glutamate transporters in the hippocampus of rats subjected to maternal separation, which is another model of early life stress. In contrast, VGLUT1 expression was unchanged in the ventral hippocampus of PRS rats, thus excluding a reduced glutamate transport into synaptic vesicles. Our data strongly suggest that PRS causes a long-lasting dysfunction in the intrinsic machinery controlling exocytotic glutamate release in the ventral hippocampus. Regulated neurotransmitter release depends on Ca^{2+} sensors, C2 domain proteins that associate with phospholipids, the three proteins of the SNARE complex (VAMP, SNAP25, syntaxin), and other proteins regulating the trafficking of synaptic vesicles, such as synaptophysin, synapsins, munc-18, and Rab3A (for review, see Han et al., 2010; Epp et al., 2011; Hussain and Davanger, 2011; Walter et al., 2011). Synaptophysin acts as a regulator of the SNARE complex (Hinz et al., 2001), and is also considered as a marker protein of presynaptic nerve endings (Thome et al., 2001; Grillo et al., 2005). Synapsins are involved in the clustering of synaptic vesicles to the reserve pool near the release sites in presynaptic terminals (Valtorta et al., 1992; Greengard et al., 1993). Munc-18 is a molecular chaperone

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Table 2. EPM data of control and PRS rats bilaterally infused with vehicle or CGP53432 plus LY341495 into the ventral hippocampus

	CONT/vehicle	PRS/vehicle	CONT/CGP + LY	PRS/CGP + LY
Number of entries into the open arm	5.5 ± 0.6	2.3 ± 0.5[a]	5.0 ± 0.4	4.3 ± 0.2
Number of entries into the closed arm	8.2 ± 0.5	7.0 ± 0.6	11 ± 1.7	9.2 ± 0.7
Number of crossings through the center	13 ± 0.8	11 ± 1.7	13 ± 1.2	12 ± 1.0
Episodes of head dips	11 ± 1.5	4.8 ± 1.4[b]	14 ± 1.4	9.7 ± 1.3[c]
Rearing episodes	10 ± 1.5	8.8 ± 0.5	8.5 ± 1.6	10 ± 0.9
Latency to enter the open arm	6.2 ± 1.4	71 ± 25[d]	5.9 ± 1.4	14 ± 2.9
Latency to enter the closed arm	37 ± 4.2	1.9 ± 0.5[e]	57 ± 15	12 ± 4.9

Data were obtained from the same control (CONT) and PRS rats of Figure 5*B*; see legend for details on treatments with CGP53432 and LY341495. Values are means ± SEM of six rats per group. [a]$p < 0.01$ versus all other groups; [b]$p < 0.01$ versus CONT/vehicle and CONT/CGP + LY, and $p < 0.05$ versus PRS/CGP + LY; [c]$p < 0.05$ versus CONT/CGP + LY and PRS/vehicle; [d]$p < 0.01$ versus all other groups; and [e]$p < 0.05$ versus CONT/vehicle and $p < 0.01$ versus Cont/CGP + LY. CONT, controls.

of syntaxin-1, which is involved in mechanisms of SNARE-mediated membrane fusion and docking of large dense-core vesicles to the plasma membrane (Han et al., 2010). Rab3A, a member of a large family of monomeric GTP-binding proteins, regulates the trafficking of synaptic vesicles and cooperates with synapsin II in promoting the latest steps of neurotransmitter release (Sakane et al., 2006; Coleman and Bykhovskaia, 2010). PRS caused large reductions in the levels of all these proteins (except SNAP25) in the ventral hippocampus, and only reductions in the levels of syntaxin and synapsin Ia/b in the dorsal hippocampus. This profile of expression of vesicle-associated proteins fits nicely with the finding that glutamate release was reduced in the ventral hippocampus, but not in the dorsal hippocampus of PRS rats.

A potential consequence of the reduced glutamate release in the ventral hippocampus is that PRS rats become refractory to paroxysmal activity sustained by an enhanced release of glutamate. In release experiments, we used kainate as an alternative to high concentrations of K^+. Kainate acting at presynaptic receptors is known to either stimulate or depress glutamate and GABA release depending on the concentrations and the hippocampal subregions (Ferkany et al., 1982; Poli et al., 1985; Chittajallu et al., 1996; Schmitz et al., 2001; Rodríguez-Moreno and Sihra, 2004). In our synaptosomal preparations, kainate caused a large release of glutamate, which was blunted in the ventral hippocampus of PRS rats. PRS rats were highly resistant to kainate-induced limbic motor seizures, which model temporal lobe epilepsy in humans (Ben-Ari and Cossart, 2000; Coulter et al., 2002). All PRS rats treated with kainate showed only mild motor signs, and none of them developed the typical secondarily generalized limbic motor seizures, which were instead seen in control rats. However, the relationship between early life stress and kainate-induced seizures is uncertain because a single episode of restraint stress on gestational day 18 enhanced kainate-induced seizures in adult gonadectomized offspring (Frye and Bayon, 1999), whereas treatment with β-methasone on gestational day 15 reduced the susceptibility to fluorothyl-induced clonic seizures, but not to kainate-induced seizures, at postnatal day 15 (Velíšek, 2011).

PRS had profound effects on glutamate release, but it failed to affect GABA release in the ventral hippocampus. The lack of changes in VGLUT1 expression and D-[^{3}H]-aspartate uptake excluded that the number of glutamatergic nerve terminals was reduced in the ventral hippocampus of PRS rats. Reductions in munc-18 and Rab3A might provide some specificity for glutamate versus GABA release. Accordingly, munc-18 regulates the size of the readily releasable vesicle pool in glutamatergic but not GABAergic terminals (Augustin et al., 1999), and Rab3A is preferentially, albeit not exclusively, expressed in glutamatergic terminals (Geppert et al., 1994). Our data suggest that PRS causes an imbalance between excitatory and inhibitory neurotransmission in the ventral hippocampus, an effect that might perturb cognitive functions related to stress and emotions (for review, see Bannerman et al., 2004; Engin and Treit, 2007; Fanselow and Dong, 2010). Presynaptic alterations in the glutamate/GABA balance have been associated with anxiety, depressive-like behavior, and memory impairment (Tordera et al., 2007; Garcia-Garcia et al., 2009; Chen et al., 2010). Thus, the imbalance between excitatory and inhibitory neurotransmission in the ventral hippocampus might contribute to explain the anxious/depressive-like phenotype of PRS rats (Vallée et al., 1997; Zuena et al., 2008; Morley-Fletcher et al., 2011; see also present data).

Another important aspect of our study is the regional specificity in the reduction of glutamate release seen in PRS rats. Previous studies have shown that stressors of various types can have profound effects on glutamatergic transmission not only in the hippocampus but also in the prefrontal cortex, striatum, and amygdala (Fumagalli et al., 2009; Mozhui et al., 2010; Uchida et al., 2011; Farley et al., 2012; for review, see Popoli et al., 2012). For example, Fumagalli et al. (2009) have found that PRS rats challenged with a swim test in adulthood showed an attenuated phosphorylation of the NR1 subunit of NMDA receptors in the prefrontal cortex, but not in the hippocampus. In our PRS rats, glutamate release was reduced in the ventral hippocampus, but not in the dorsal hippocampus, prefrontal cortex, perirhinal cortex, striatum, or amygdala. The specificity for ventral versus dorsal hippocampus is in agreement with previous data showing that group-I mGlu receptor signaling is selectively blunted in the ventral hippocampus of PRS rats (Zuena et al., 2008). Also, the lack of changes in glutamate release in the dorsal hippocampus is in agreement with the evidence that PRS rats do not show abnormalities in spatial memory unless they are >10 months of age (Vallée et al., 1999), when changes in the expression of postsynaptic mGlu receptors are prominent (Van Waes et al., 2009).

To examine whether a causal relationship exists between reduction of glutamate release in the ventral hippocampus and anxiety-like behavior in PRS rats, we performed microinfusion studies with a mixture of mGlu2/3 and $GABA_B$ receptor antagonists, which was proven to selectively enhance glutamate release in the ventral hippocampus. All animals selected for this experiment had been tested for anxiety-like behavior ~2 weeks before intrahippocampal microinfusions. Following infusions with vehicle or CGP52432 and LY341495, we designed a behavioral protocol based on two consecutive tests in the light–dark box (first) and the EPM (immediately after). This is unusual because repetitive tests for the assessment of anxiety-like behavior are generally performed with at least 1 week of interval to avoid the influence of the previous test experience (Võikar et al., 2004; Cryan and Holmes, 2005; Paylor et al., 2006; Ballaz et al., 2007) and treatment-dependent fluctuations in behavior that may occur between two consecutive tests (Izídio et al., 2005; Ramos, 2008). We adopted the strategy of two consecutive behavioral tests to avoid the need

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to reinject CGP52432 and LY341495 in the ventral hippocampus without having knowledge of the neuroplastic changes induced by these drugs in the hippocampus. This may confound the interpretation of the EPM data (but not the interpretation of the light–dark box data) following injection of CGP52432 and LY341495 in unstressed and PRS rats. Taking into account these possible limitations, our data suggest a causal relationship between reduction of glutamate release in the ventral hippocampus and anxiety-like behavior in PRS rats. The doses of CGP52432 and LY341495 we have used (1 and 100 pg, respectively), did not cause nonspecific changes in motor activity in the light–dark box and EPM, and did not affect anxiety-like behavior in unstressed control rats. Thus, pharmacological enhancement of glutamate release in the ventral hippocampus could specifically reverse anxiety-like behavior in PRS rats.

The mechanisms by which PRS causes a dysfunction in glutamate release in the ventral hippocampus is unknown. PRS rats are characterized by a hyper-reactivity of the hypothalamic—pituitary–adrenal axis, which results into a prolonged corticosterone response to stress (Maccari et al., 1995), and this might have a causal role in the dysfunction of glutamate release in the ventral hippocampus (Popoli et al., 2012). The hypothesis that high levels of corticosterone cause a long-lasting reduction in glutamate release in PRS rats warrants further investigation. We cannot exclude that changes in glutamatergic neurotransmission occurring in other brain regions contribute to the anxiety-like phenotype of PRS rats. Our data suggest that an impairment of glutamate release in the ventral hippocampus may lie at the core of the neuroplastic program induced by PRS, and strongly correlates with the development of anxiety-like behavior in these rats.

In conclusion, these findings support the "glutamatergic hypothesis" of depression and anxiety (Maeng and Zarate, 2007; Matrisciano et al., 2007; Hashimoto, 2009; Popoli et al., 2012), and suggest to extend the study of the balance between excitatory and inhibitory neurotransmission in the ventral hippocampus in other putative animal models of anxiety to develop new therapeutical strategies for stress-related disorders.

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-CHAPTER TWO-

The effects of antidepressant drugs on glutamate-related disorders

3. Chronic treatment with antidepressants reverses the deficit in glutamate release and glutamate-related behavioral alterations induced by prenatal stress

Data from Chapter one highlight the role of hippocampus in the pathophysiology of the anxiety-like phenotype induced by PRS. In particular, the reduction of glutamate release in the ventral hippocampus is likely to be causally related to this "anxious" behavior. Anxiety disorders show high comorbidity with depression, and both diseases implicate brain structures involved in emotion, such as amygdala and hippocampus (Mineka *et al.*, 1998; Kaufman *et al.*, 2000). Interestingly, since stress is a major risk factor for anxiety and depression, it has been proposed to revisit the criteria of DSM IV to include these disorders in a section entitled "Trauma and Stressor-Related Disorders" (Friedman *et al.*, 2011). Moreover, findings suggest that antidepressants are useful first-line agents for most of the anxiety disorders (Ravindran and Stein, 2010). For example, Tianaptine shows both anxiolytic and antidepressant activity and, more interestingly, recent evidence supports its role on glutamatergic neurotransmission (McEwen, 2010). Emerging data show that normalizing and stabilizing glutamate neurotransmission is also a potential target of drugs treating depressive disorders (Bonanno *et al.*, 2005; Sanacora *et al.*, 2012).

Thus , we wanted to assess whether the PRS glutamatergic phenotype could be restored by chronic treatment with agomelatine and fluoxetine; then, we evaluated glutamate release and synaptic vesicle-associated proteins in hippocampus as such as anxiety-like and depressive-like behaviors in the light-dark test and forced swim test, respectively.

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Manuscript in preparation

Chronic treatment with antidepressants reverses the deficit in glutamate release and glutamate-related behavioral alterations induced by prenatal stress in rat

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Abstract

Abnormalities of synaptic transmission and plasticity in the hippocampus represent an integral part of the altered programming triggered by early life stress which results into increased vulnerability to stress-related and neuropsychiatric disorders in the adult life. Prenatally restraint stressed (PRS) rats develop long-lasting biochemical and behavioral changes, which are expression of an anxious/depressive-like phenotype. Most of the behavioral and neurochemical alterations induced by PRS can be reversed by treatment with different classes of antidepressant. In addition, most of the abnormalities in synaptic transmission and plasticity in the hippocampus of PRS rats are seen in the ventral hippocampus, the specific portion of the hippocampus that encodes memories related to stress and emotions. We have recently proven a causal link between a selective impairment of glutamate release in the ventral hippocampus associated with large reductions in the levels of SNARE-related proteins and enhanced anxiety behavior observed in male PRS rats. We thus decided to explore the possibility of reversal of glutamate deficit by antidepressant treatment in PRS rats and treated animals for three weeks with fluoxetine, a classic SSRI, or agomelatine, a novel antidepressant that behaves as a MT1/MT2 melatonergic receptor agonist and 5-HT2C receptor antagonist. We report that chronic antidepressant treatment, in particular with agomelatine, fully restored the deficit in glutamate release induced by PRS. This was associated to an increase in SNARE associated proteins and a reduction in anxious/depressive-like behavior. These findings indicate that an impairment of glutamate release in the ventral hippocampus is a key component of the neuroplastic program induced by PRS, and support the the "glutamatergic hypothesis" of depression and anxiety. At the same time, our data confirm the predictive value of the PRS model, lending credit to the hypothesis that depression and anxiety develop as late consequences of a pathological programming triggered early in life.

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Introduction

Stressful life events impact on memory and emotional responses and are known to precipitate mood/anxiety disorders (McEwen, 2012; Meaney *et al.*, 2007; Darnaudery and Maccari, 2008; Lupien *et al.*, 2009). In the last few years, numerous lines of evidence accumulated in favor of a role for glutamate in psychiatric pathophysiology (Altamura *et al.*, 1993; Drevets 2000, 2004; Sanacora *et al.*, 2004). As a consequence, now several compounds are under development for the treatment of mood disorders (depression, bipolar disorder, anxiety) that modulate glutamate receptors or neurotransmission at various levels (Holden, 2003). Some of these putative drugs may work by stabilizing glutamate release when its synaptic level becomes too high, a feature that is now considered to be part of the pathophysiology of mood disorders (Sapolsky, 2000; Zarate *et al.*, 2002). A few studies in rodents have suggested that chronic antidepressant treatments reduce glutamate release in limbic/cortical areas in basal conditions (Bonanno *et al.*, 2005; Popoli *et al.*, 2012). In particular, chronic antidepressants reduce (K depolarization-evoked release of endogenous glutamate from synaptic terminals of HPC, with concomitant modifications in protein-protein interactions regulating assembly of the presynaptic SNARE complex, that mediates fusion of synaptic vesicles. Hence, glutamate-based therapies might represent an effective alternative to biogenicamine-based agents for depression. Glutamate neurotransmission was recently implicated in the action of acute stress in adult rats and in antidepressant mechanisms (Musazzi *et al.* 2010; Reagan *et al.* 2012; Tardito *et al.* 2012). However, there are no data concerning the glutamate transmission in the action of antidepressant mechanisms in experimental paradigms of chronic stress and in particular early life stress.

We have recently proven (Marrocco *et al.*, 2012) the first evidence that early life stress directly affects glutamate release and its presynaptic machinery in animal model that recapitulates some features of depression such has the prenatally stressed rat (PRS). PRS male rats show behavioral, cellular, and biochemical abnormalities which are consistent with an anxious/depressive phenotype and present life span alterations in the glutamate machinery (Darnaudery & Maccari, 2008; Zuena *et al.*, 2008; Laloux *et al.*, 2012; Marrocco *et al.*, 2012). In addition, most of the abnormalities in synaptic transmission and plasticity in the hippocampus of PRS rats are seen in the ventral hippocampus (Zuena *et al.*, 2008; Morley-Fletcher *et al.*, 2011), the specific portion of the hippocampus that encodes memories related to stress and emotions (Fanselow and Dong, 2010). Male PRS rats display a selective impairment of glutamate release in the ventral hippocampus associated with large reductions in the levels of SNARE-related proteins and enhanced anxiety behavior, and pharmacological enhancement of glutamate release in the ventral hippocampus (intahippocampal injection with a cocktail containing a mGlu2/3 receptor antagonist *plus* a GABA-B receptor antagonist) corrected their anxious-like phenotype (Marrocco *et al.*, 2012).

Since most of the behavioral and neurochemical alterations induced by PRS can be reversed by treatment with different classes of antidepressant (Morley-Fletcher *et al.*, 2003, 2004, 2011; Mairesse *et al.*, 2012), and taking into account the emerging "glutamatergic" hypothesis of depression and anxiety, (Holden *et al.* 2003; Bonanno *et al.* 2005; Maeng and Zarate 2007; Hashimoto *et al.*, 2009; Popoli *et al.*, 2012), we decided here to explore the possibility of reversal of glutamate deficit by antidepressant treatment in PRS rats. In order to asses it animals were chronically treated with fluoxetine, a classic SSRI, or agomelatine, a novel antidepressant that behaves as a MT1/MT2 melatonergic receptor agonist and 5-HT2c receptor antagonist and that has proven its efficacy in restoring neuroplastic impairments in the hippocampus, sleep-wake cycle and anxious/depressive like features in PRS rats (Morley-Fletcher *et al.*, 2011; Mairesse *et al.*, 2012). We report that chronic antidepressant treatment, in particular with agomelatine, fully restored the deficit in glutamate release induced by PRS. This was associated to an increase in SNARE associated proteins and a reduction in anxious/depressive-like behavior.

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Methods

Animals. Forty nulliparous female Sprague-Dawley rats (20 for control and 20 for PRS groups), weighing approximately 250g, were purchased from a commercial breeder (Charles River, France). Animals were housed at constant temperature (22±2°C) and under a regular 12hr light/dark cycle (lights on at 8.00 a.m.). Pregnant females were randomly assigned to stressed or control groups. (n=12 per group).

Stress protocol. Animals were subjected to PRS according to our standard protocol (Maccari *et al.*, 1995; Morley-Fletcher *et al.*, 2003). From day 11 of pregnancy until delivery, pregnant female rats were subjected to three stress sessions daily (45 min. each), during which they were placed in transparent plastic cylinders and exposed to bright light. Only male offspring from litters containing 10-14 pups with a comparable number of males and females were used for the experiments. A maximum of one or two male pups were taken from each litter for each measure to remove any litter effects (Becker and Kowall, 1977; Chapman and Stern, 1979). All experiments followed the rules of the European Communities Council Directive 86/609/EEC. The local ethical committee approved the prenatal stress procedure.

Antidepressant treatment. Antidepressant drugs were dissolved in hydroxyethylcellulose (HEC 1% suspension in distilled water). Rats were treated daily during three weeks with i.p. injections of fluoxetine (5 mg/mL/kg, Sigma), agomelatine (40 mg/2ml/kg, Servier, France) or 1 ml/kg of HEC alone (vehicle). The dose of agomelatine was selected on the basis of previous reports (Van Reeth *et al.* 1997; Papp *et al.* 2003; Banasr *et al.* 2006; Soumier *et al.* 2009) and and on previous data obtained in the PRS rats (Morley-Fletcher *et al.*, 2011). Injections were performed 2 h prior to the onset of the dark phase of the 12-h light/dark cycle, based on the circadian rhythm resynchronization properties and antidepressant activity of agomelatine (Van Reeth *et al.* 1997; Papp *et al.* 2003).

Glutamate and GABA release experiments. Purified synaptosomes isolated from the ventral hippocampus were prepared essentially according to Dunkley and coworkers (1986), with minor modifications. Briefly, the tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with TRIS (final concentration 0.01 M) using a glass Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged at 1000 x *g* for 5 min, to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll gradient (6%, 10% and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 x *g* for 5 min. The layer between 10% and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. The synaptosomal pellet was then resuspended in physiological medium (standard medium) having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10 mM, glucose, 10; pH 7.2-7.4. Synaptosomes were incubated for 15 min a 37 °C in a rotary water bath and superfused at 0.5 ml/min with standard physiological solution. When studying the release of neurotransmitter evoked by high concentrations of K^+, synaptosomes were transiently (90 s) exposed, at t = 39 min, to 12 mM K^+ (substituted for NaCl in the superfusate). Superfusion was always performed with media containing 50 µM amino-oxyacetic acid (Sigma) to inhibit GABA metabolism. Three superfusate fractions were collected according to the following scheme: two 3-min fractions (basal release), one before (t = 36-39 min, b1) and one after (t = 45-48 min, b3) a 6-min fraction (t = 39-45 min; evoked release, b2). Fractions collected and superfused synaptosomes were counted for endogenous aminoacid content. Endogenous glutamate and GABA were measured by HPLC analysis after pre-column derivatization with *o*-phthalaldehyde and separation on a C_{18} reverse-phase chromatographic column (10 X 4.6 mm, 3µm; at 30° C; Chrompack, Middleburg, The Netherlands) coupled to fluorimetric detector (excitation wavelength, 350 nm; emission wavelength, 450 nm).

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Buffers and the gradient program were as follows: solvent A, 0.1 M sodium acetate (pH5.8)/methanol, 80:20; solvent B, 0.1 M sodium acetate (pH5.8)/methanol, 20:80; solvent C 0.1 M sodium acetate (pH6.0)/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; isocratic flow , 2 min; 78% A and 22% B in 2 min; isocratic flow , 6 min; 66% A and 34% B in 3 min; 42% A and 58% B in 1 min; 100% B in 1 min; isocratic flow, 2 min; 100% C in 3 min; flow rate, 0.9 ml min^{-1}. Homoserine was used as internal standard. Synaptosomal protein contents were determined according to Bradford (1976). The amount of endogenous glutamate and GABA from synaptosomes in superfusate fractions was expressed as picomoles per milligram of protein (pmol mg^{-1} protein). The depolarization-induced overflow was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (evoked release, b2).

Western blot analysis. Following antidepressant treatment, a separate set of control and PRS rats (n = 6 per group) were killed by decapitation, the hippocampus were rapidly dissected and immunoblotting was carried on hippocampus. To isolate synaptosomes, tissue was manually homogenized with a potter in 10 volumes of HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES pH 7.4). All procedures were performed at 4°C. Homogenates were centrifuged at 1000 x g for 10 min and resulting supernatants were centrifuged at 10000 x g for 15 min. The pellet obtained from the second centrifugation was resuspended in 10 volumes of HEPES- buffered sucrose and then spin again at 10000 x g for 15 min. This pellet contained the crude synaptosomal fraction. To validate the purity of this synaptosomal fraction we used anti-histon H3, anti-β tubulin, anti-synapsin Ia/b in immunoblot analysis. BCA assay was used to determine protein concentration. Synaptosomes lysates were resuspended in laemli reducing buffer and 20µg of each sample were first separated by electrophoresis on Criterion TGX 4-15% precast SDS-polyacrylamide gels (Bio-Rad) and later transferred to nitrocellulose membranes (Bio-Rad). Transfer was performed at 4°C in a buffer containing 35mM TRIS, 192mM glycine and 20% methanol. We used the following primary antibodies: rabbit polyclonal anti-synapsin IIa (sc-25538, 1:4000), rabbit polyclonal anti-VAMP (synaptobrevin, sc-13992, 1:2000) and mouse monoclonal anti-rab3a (107111, 1:2000); mouse monoclonal anti-β-actin (A5316, 1:80000) (purchased from Sigma). Secondary anti-mouse or anti-rabbit antibodies (purchased from Amersham) were used a dilution at 1:10000.
Densitometric analysis was performed with Quantity One software (Bio-Rad) associated to a GS-800 scanner. The ratio of individual proteins to β-actin was then determined and these values were compared for statistical significance.

Behavioral analysis

Anxiety

Light and dark box. Anxiety like behavior was assessed on day 15 of chronic antidepressant treatment in the light and dark test as previously described (Marrocco *et al.*, 2012). The light and dark box setup consisted of two compartments: one black (30 x 32 x 32 cm, 5 lux) and one white (45 x 32 x 32 cm, 50 lux) connected via a small opening (10 x 15 cm) enabling transition between the two boxes. Adult male rats (n=9 rats per group) were placed in the white chamber of the apparatus and the latency (in s) to the first entry into the white box after during the 5 min. test was assessed *via* a video camera placed above the box. Behavior was manually analyzed (Observer 20 Noldus, Wageningen, The Netherlands).

Depression

Forced swim test. Depression-like behavior was assessed testing the animals in the forced swim test on day 16 of chronic antidepressant treatment. Briefly, animals (n=9 per group) were subjected to an

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adapted version of the forced swim test (Porsolt *et al.* 1978) in a cylindrical container (height=59 cm; diameter=25 cm) filled with water at 25°C up to a level of 36 cm. The test was carried out between 12:00 and 17:00 h. Twenty-four hours after a 15-min session (pre-test, on day 15), control and PRS rats were tested (day 16) for a 5-min session during which immobility latency and duration, climbing, and swimming were automatically analyzed using a video tracking software (View Point, France).

Social memory. The juvenile recognition abilities of the rat were assessed using a procedure adapted from Angelmann *et al.* (1995), after 17 days of treatment with agomelatine or fluoxetine. Rats (n=9 rats per group) were individually placed in transparent cages for 5 min. for habituation. During each of the three sessions, a given male juvenile (2 mo) was introduced into the adult cage for 5 min. Then, the juvenile was removed, kept individually in a cage (27 x 21 x 14 cm) with fresh bedding; food and water were provided *ad libitum* for a defined inter-exposure interval of 30 or 120 min. The juvenile was then presented again to the tested rat for a 5 min. session. Sessions were video-recorded and the total investigatory behavior (s) of the adult toward the juvenile (time spent grooming, licking, anogenital interaction, sniffing, playing) was measured by a trained observer (Observer 20 Noldus, Wageningen, The Netherlands).

Statistical analysis

Data of release experiments, immunoblot analysis, and behavioral data with light-dark box, forced swim test and social memory were analyzed by two-way ANOVA (group x treatment) followed by the Neumann-Keuls post-hoc or planned comparisons. A p value <0.05 was considered as statistically significant.

Results

Chronic antidepressant treatment normalizes the reduced depolarization-evoked release of glutamate in the ventral hippocampus of PRS rats

To study the effects of antidepressant treatment on neurotransmitter release in PRS rats, we used superfused synaptosomes prepared from adult male PRS rats and their age-matched controls. Our superfusion method eliminates the components in neurotransmitter release mediated by the inverse operation of membrane transporters and the influence of endogenous ligands acting at presynaptic receptors, thus allowing a reliable estimation of how the intrinsic release machinery responds to depolarization or other stimuli (Raiteri *et al.* 1974; Raiteri and Raiteri, 2000). Depolarization-evoked release in this system is exocytotic and entirely depended on extracellular Ca^{2+} (Bonanno *et al.*, 2005). Synaptosomes prepared from the ventral hippocampus, from control or PRS rats were challenged with depolarizing concentrations of K^{+}, and the superfusate was used for measurements of endogenous glutamate and GABA release. Depolarization-evoked glutamate release was largely reduced in the ventral hippocampus of PRS vehicle-treated rats, and agomelatine treatment and fluoxetine reversed this profile (ANOVA group by treatment, $F_{(2,24)} = 13.14$, $p < 0.01$) (**Fig. 1A**). Neither basal nor evoked GABA release differed between PRS and control rats before and after antidepressant treatment (**Fig 1B**).

Effect of chronic antidepressant treatment on the expression of SNARE-associated proteins in the hippocampus of PRS rats

We measured the levels of synaptic-vesicle associated proteins in purified synaptosomal membranes prepared from the hippocampus of control and PRS rats (**Fig. 2**). Substantial reductions in the levels of Rab3A (ANOVA group by treatment $F_{(2,18)} = 11,46$, $p < 0.01$), VAMP (synaptobrevin) (ANOVA group by treatment, $F_{(2,18)} = 6.36$, $p < 0.01$), and synapsin IIa (ANOVA group by treatment, $F_{(2,18)} =$

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16.12, $p < 0.01$ p<0.01) were found in the ventral hippocampus of PRS vehicle-treated rats. Antidepressant treatment with agomelatine increased the levels of all three proteins in PRS rats.

Antidepressant treatment with agomelatine reduces anxious/depressive-like behavior in PRS rats and improves emotional memory

PRS rats show anxiety-like behavior and increased immobility in the forced swim test (Vallée *et al.* 1997; Zuena *et al.* 2008; Morley-Fletcher *et al.* 2003; 2004; 2011), and the ventral portion of the hippocampus is involved in emotion and anxiety (Fanselow and Dong, 2010). Both control and PRS rats used for measurements of glutamate release in synaptosomes (see above) had been tested for anxiety-like behavior in the light and dark test. PRS rats treated with vehicle displayed an increased latency to enter the light compartment of the box (ANOVA, group x treatment $F_{(2,37)} = 3.01$, $p < 0.05$) (**Fig. 3 A**) and agomelatine treatment reversed this profile, whereas fluoxetine was slightly effective (p=0.07). When tested for depressive-like behavior in the forced swim test, PRS vehicle–treated rats spent more time in immobility and agomelatine treatment, but not fluoxetine, reversed this profile. (ANOVA, group x treatment, $F_{(2,38)} = 3.82$, $p < 0.05$) (**Fig. 3B**). Finally, we extended the study to investigate social discrimination capabilities toward a juvenile. PRS induced inability in adult animals to recognize a juvenile after an interexposure interval of 120 min while control animals were able to recognize the previously exposed juvenile 30 and 120 min after the first exposure. Antidepressant treatment with agomelatine markedly improved social memory in PRS rats which reduced their investigation of the juvenile 120 min after the first exposure, whereas fluoxetine was ineffective (ANOVA group x treatment x interval exposure, $F_{(2,99)} = 2.99$, $p < 0.05$) (**Fig 3C**).

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Discussion

We have shown for the first time the efficacy of antidepressant treatment on glutamate neurotransmission and anxious/depressive like behavior in a model that replicates developmental factors involved in the etiology of anxious/depressive disorders (reviewed by Krishnan and Nestler, 2010), and allows to examine the outcome of early life stress in the adult. Prenatal restraint stress, which is a model that recapitulates some of the features of depression and anxiety, causes a selective impairment of glutamate release in the ventral hippocampus, a brain region which specifically encodes memories related to stress and emotions (Fanselow and Dong, 2010). PRS had profound effects on glutamate release, but it failed to affect GABA release in the ventral hippocampus. Our data suggest that PRS causes an imbalance between excitatory and inhibitory neurotransmission in the ventral hippocampus, an effect that might perturb cognitive functions related to stress and emotions (reviewed by Bannerman *et al.* 2004; Engin and Treit, 2007; Fanselow and Dong, 2010). Presynaptic alterations in the glutamate/GABA balance have been associated with anxiety, depressive-like behavior, and memory impairment (Tordera *et al.*, 2007; Garcia-Garcia *et al.*, 2009; Chen *et al.* 2010). Thus, the imbalance between excitatory and inhibitory neurotransmission in the ventral hippocampus might contribute to explain the anxious/depressive-like phenotype of PRS rats (Vallée *et al.* 1997; Zuena *et al.* 2008; Morley-Fletcher *et al.* 2011; Marrocco *et al.*, 2012). The mechanisms by which PRS causes a dysfunction in glutamate release in the ventral hippocampus is unknown. PRS rats are characterized by a hyper-reactivity of the hypothalamic-pituitary-adrenal axis, which results into a prolonged corticosterone response to stress (Maccari *et al.* 1995), and this might have a causal role in the dysfunction of glutamate release in the ventral hippocampus (Popoli *et al.* 2012). The hypothesis that high levels of corticosterone cause a long-lasting reduction in glutamate release in PRS rats warrants further investigation. The present study and a recent work (Marrocco *et al.* 2012) indicate that an impairment of glutamate release in the ventral hippocampus lies at the core of the neuroplastic program induced by PRS, and strongly correlates with the development of anxiety-like behavior in these rats. Both agomelatine and fluoxetine, two antidepressants with different mechanism of action corrected glutamate neurotransmission while only agomelatine normalized SNARE associated proteins in PRS rats, suggesting that the drugs impact mechanisms that lie at the core of the maladaptive programming induced by PRS. To our knowledge this is the first evidence of reversal of a glutamatergic impairment and glutamate-related behavior in an animal model of early life stress, since the efficacy of fluoxetine and agomelatine on glutamate release was reported only in basal conditions or following acute adult stress exposure (Bonanno *et al.* 2005; Musazzi *et al.* 2010; Reagan *et al.* 2012; Tardito *et al.* 2012). Agomelatine in particular, was very effective in correcting the neurochemical, as well as the behavioral alterations in PRS rats with no or poor effect in control animals. Indeed agomelatine reduced anxiety, immobility behavior and it improved social discrimination (memory effect) in PRS rats. The specific effect of agomelatine on PRS rats has already been reported (Morley-Fletcher *et al.* 2011) with agomelatine, acting as an etiopathogenetic drug with its action specific to the pathological state (i.e., agomelatine behaves as a "disease-dependent" drug). Also, the greater efficacy of agomelatine with respect to fluoxetine in correcting behavioral alterations in PRS can be explained by a more selective action of this antidepressant in the ventral hippocampus of PRS rats a brain region which specifically encodes memories related to stress and emotions. Indeed in PRS rats, agomelatine preferentially enhanced neurogenesis in the ventral hippocampus, an effect which is consistent with the reversal of anxiety behavior of these animals, while action of fluoxetine is less region specific (Morley-Fletcher *et al.* 2011). The anxiolytic effects of agomelatine in the present study are consistent with recent data showing its efficacy in general anxiety disorder in clinical trials (Stein *et al.*, 2008; Stein *et al.*, 2012).

In the present study, agomelatine treatment reduced anxiety behavior in PRS rats as well it improved their ability to recognize a conspecific juvenile, thereby suggesting that changes in anxiety-related behavior may be associated with changes in social interaction (see also Landgraf *et al.*, 1995).

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Although the relationship between social memory and the particular emotionality is far from clear, the notion is attractive, that in the end the effects on emotion influence social memory by changing the balance between reserve and explorative curiosity. In this line, there is evidence that reduced anxiety is associated with improved cognitive flexibility (Blazevic *et al.* 2012). This balance, however, is certainly more important during social interaction among adult animals than during exposure to a juvenile. In support of this prenatal stress is known to impair in the long term social behavior by reducing social play in juveniles (Takahashi *et al.* 1992; Morley-Fletcher *et al.* 2003) and reduced quantity and quality of social interaction at the adult stage (Lee *et al.* 2007). At the same time PRS animals display long term cognitive alterations. Thus, the impairment in social memory observed in our animals fits well with their anxious phenotype.

In conclusion, our data demonstrate that impairment in the glutamate machinery is a key component of the altered programming triggered by PRS, and that chronic antidepressant treatment in particular with agomelatine corrected the enduring pathological changes in glutamate neurotransmission and glutamate-related behaviour that develop in response to PRS. These findings support the the "glutamatergic hypothesis" of depression and anxiety (Holden et al; 2003; Bonanno *et al.* 2005; Maeng and Zarate 2007; Matrisciano *et al.* 2007; Hashimoto *et al.* 2009; Popoli *et al.* 2012) and at the same time, they confirm the predictive value of the PRS model, lending credit to the hypothesis that depression and anxiety develop as late consequences of a pathological programming triggered early in life.

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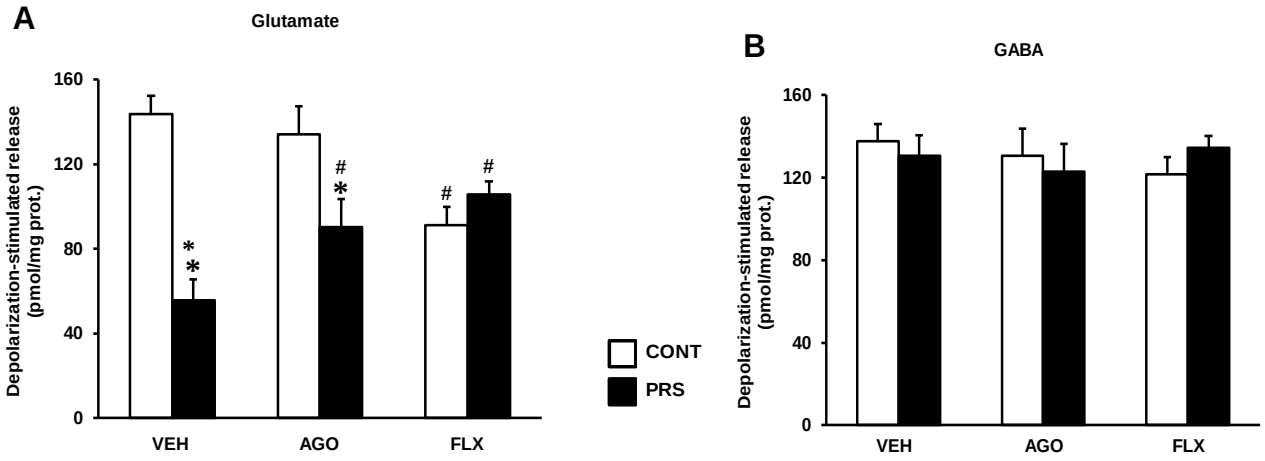

Fig. 1 - Superfused synaptosomal preparations from ventral hippocampus, of control or PRS rats (both treated with vehicle=VEH, agomelatine=AGO or fluoxetine=FLX) were stratified at the bottom of superfusion chambers (three superfusion chambers for each synaptosomal preparation) and superfused as described in the Method section. At t=39 min of superfusion, synaptosomes were challenged with 12 mM K+. Synaptosomes were used for measurements of glutamate (A), or GABA (B) release. Data are expressed as K+-induced overflow. Glutamate and GABA overflow is expressed as pmoles/mg prot. Values are means ± S.E.M. of 6 experiments run in triplicate $p<0.01$ or $p<0.05$ vs. the respective control values (*) or vs. the respective values treated with vehicle (#).

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Fig. 2 Marrocco *et al.*

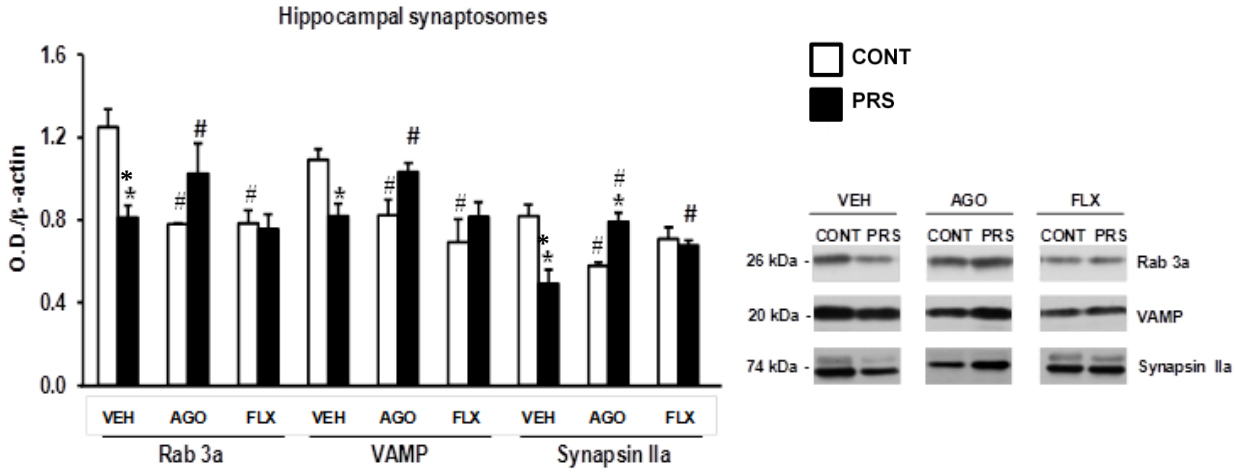

Fig. 2 - Immunoblot analysis of SNAREs, vesicle-associated proteins in synaptosomal fractions collected from hippocampus of adult PRS and control male rats, both treated with vehicle=VEH, agomelatine=AGO or fluoxetine=FLX. Values are means ± S.E.M. of 6 rats per group $p<0.01$ or $p<0.05$ vs. the respective control values (*) or vs. the respective values treated with vehicle (#).

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Fig.3 Marrocco *et al.*

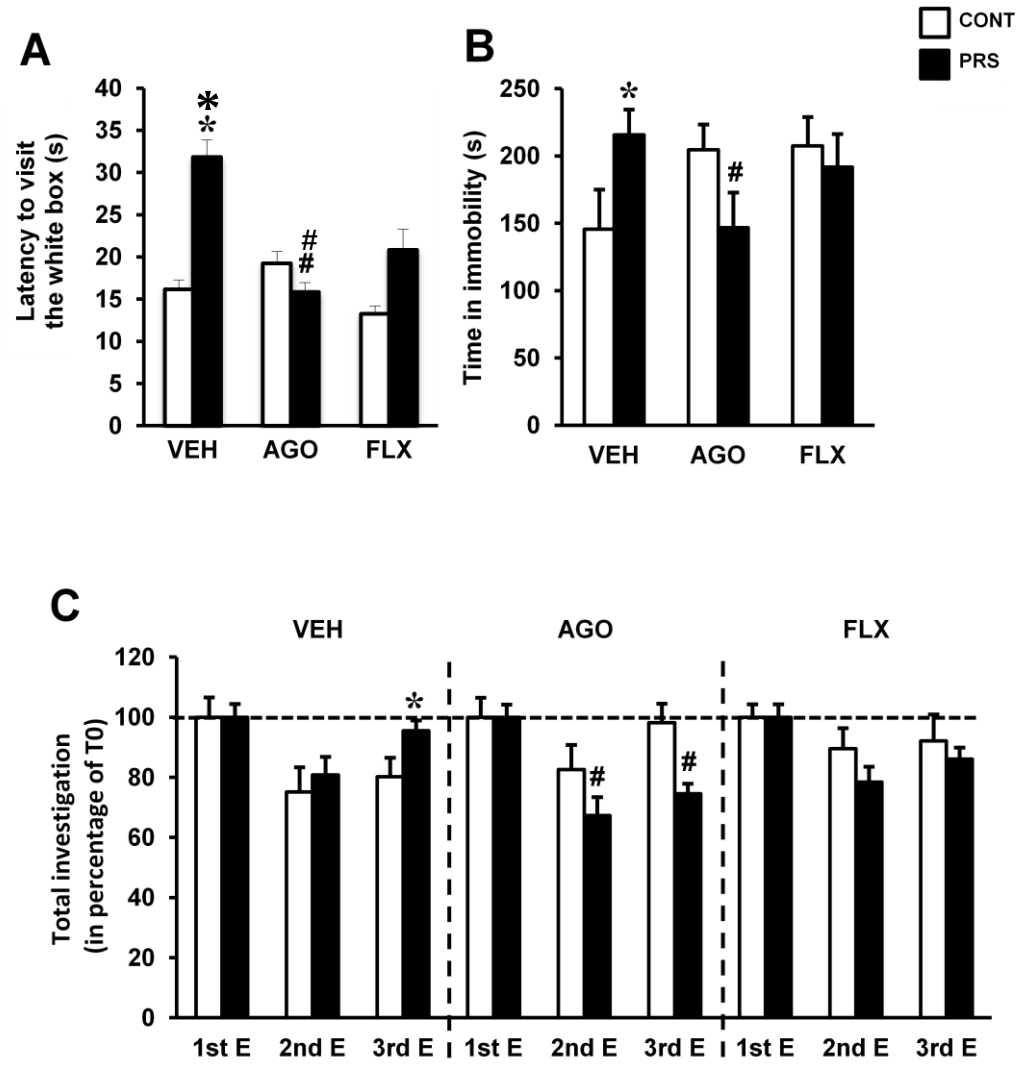

Fig. 3 – Anxiety-like behavior and depressive-like behavior are shown in (A) and (B), respectively, where the latency to visit the white box in the light-dark test and the time in immobility in the forced swim test (FST) are shown. The same groups of rats were used for behavioral analysis in the light-dark box and FST. (C) Social memory is represented, where total investigation in percentage of T0 (1st Exposure(E)) is shown. Values are means ± S.E.M. of 9 rats per group, $p<0.01$ or $p<0.05$ vs. the respective control values (*) or vs. the respective values treated with vehicle (#). vehicle=VEH, agomelatine=AGO, fluoxetine=FLX.

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-CHAPTER THREE-

Opened window to motor disorders from hippocampus

4. Early life stress causes refractoriness to haloperidol-induced catalepsy

Preface

In Chapters one and two, we have seen that PRS strongly reduced glutamate transmission in the ventral hippocampus and that, at least anxiety and depressive-like behaviors, are causally related to this impairment.

Hippocampus by itself represents a complex brain network, thereby modulating the functionality of related-brain circuits. The anatomy of memory system is a promising approach to analyze hippocampal connections, especially towards striatum (Ghiglieri *et al.*, 2011). As mentioned in the introduction, the ventral portion of the hippocampus, the subiculum, receives input directly from amygdala (Pikkarainen *et al.*, 1999; Pitkanen *et al.*, 2000), which is thought to provide a basis for the transmission of affective information to the hippocampus (Packard *et al.*, 1994; Packard and Teather, 1998). In fact, two major types of information converge in the central and basolateral amygdala. The first is the sensory information, coming from the thalamus (Herkenham, 1978; Ottersen and Ben-Ari, 1979; Turner and Herkenham, 1991; Veening, 1978b) and the cortical areas in the temporal lobes (Deacon *et al.*, 1983; Ottersen, 1982) which in turn receive input from most sensory cortical areas (Turner and Zimmer, 1984; Veening, 1978a; Wyss, 1981). The second is the affective, gustatory, and visceral input from brain areas known to process visceral and affective information, such as s*ubstantia nigra* (Beckstead *et al.*, 1979), VTA and nucleus accumbens (Fallon *et al.*, 1978). Interestingly, the same nuclei have afferent projections also to striatum and back to cerebral cortex and thalamus (Kelley and Domesick, 1982; Veening *et al.*, 1980) (for further detail on striatal circuit, see Box 3). The cells of VTA project most prominently to the nucleus accumbens, olfactory tubercle as well as to the septum, amygdala, and hippocampus. This subset of projections is known as the mesolimbic dopaminergic system. Another group of cells in the medial VTA project to the prefrontal, cingulate, and perirhinal cortex, forming the pathway known as the mesocortical dopaminergic system (Kuhar *et al.*1999; Chinta and Andersen, 2005). Owing to the overlap between the mesocortical and mesolimbic dopaminergic neurons, the two systems are often collectively referred to the mesocorticolimbic system (Wise, 2004). Interestingly, animal model of depression, exhibit a generalized impairment of the mesolimbic dopamine system (Cabib and Puglisi-Allegra, 1996; Nestler and Carlezon, 2006).The mesocorticolimbic system is critically involved in

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emotion-based behavior including motivation and reward (Chinta and Andersen 2008), and it has been hypothesized that the entire network is crucial for the action of psychostimulant and antipsychotic drugs (Kuhar *et al.* 1999). For example, Lipska and Weinberger (1993) have shown that, in rats, a damage within the ventral hippocampus reduced the catalepsy in response to haloperidol, a classical antipsychotic used to treat schizophrenia worldwide. This catalepsy mimics human pharmacological parkinsonism, since it represents the detrimental consequence of the action of classical antipsychotics on striatal dopaminergic D2 receptors in the indirect pathway (Box 3). Other studies have shown that haloperidol-induced catalepsy was attenuated by glucocorticoids, theophylline or immobilization stress (Chopde *et al.*, 1995; Dijk *et al.*, 1991).

The rat model of PRS recapitulates most of the aforementioned features, since it represents a well characterized model of depression (Morley-Fletcher *et al.*, 2011) and exhibit both impairment in the ventral hippocampus (Zuena *et al.*, 2008; Marrocco *et al.*, 2012) and reduction in glucocorticoids feedback (Maccari *et al.*, 1995). Thus, we wanted to examine the role of the "prenatally stressed hippocampus" in the putative control of the pharmacodynamics of mesocorticolimbic-related structures, such as striatum. Hence, we examined the response of PRS to haloperidol-induced catalepsy.

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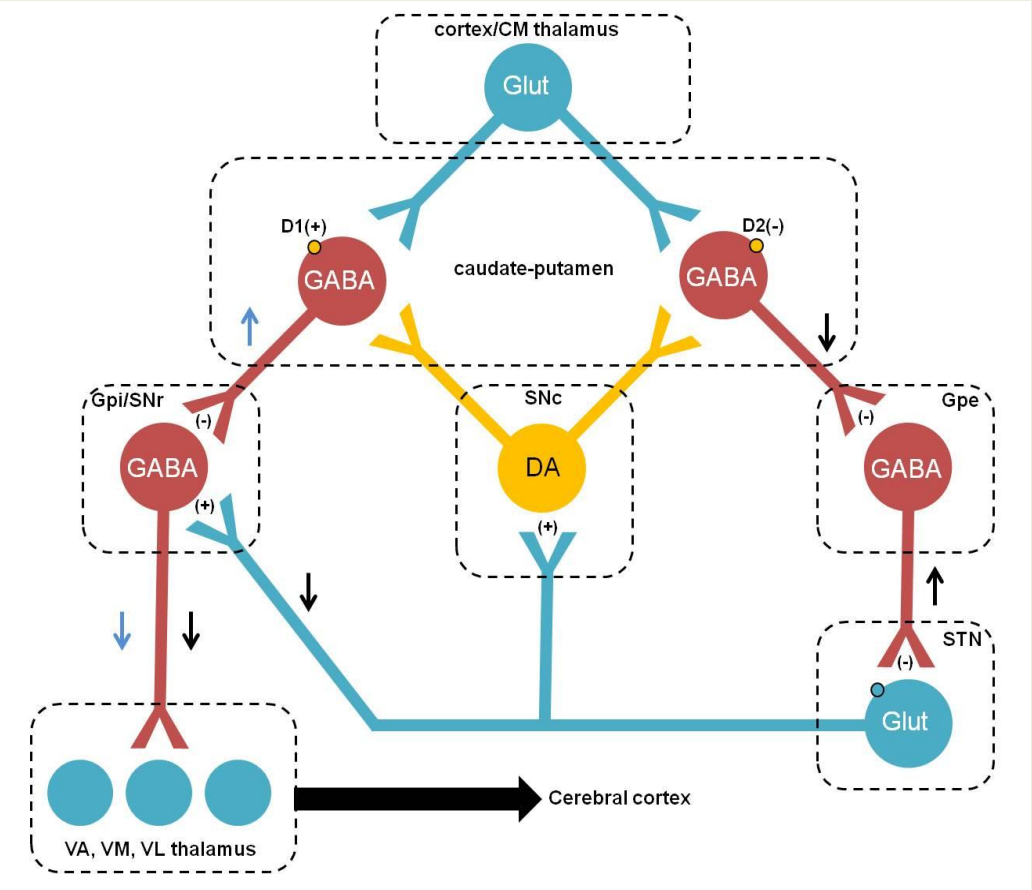
BOX 3:

Dopaminergic circuit: from *substantia nigra* to motor thalamic nuclei



Dopamine (DA) acts in the neostriatum (caudate nucleus–putamen) and influences the activity of the direct and indirect pathways (turquoise and black arrows, respectively). Both pathways converge to regulate the activity of thalamocortical neurons. The activation of D1 dopamine receptors stimulates striatal output neurons of the direct pathway, leading to inhibition of GABA neurons in the internal globus pallidus (GPi) and substantia nigra pars reticulata (SNr). In the indirect pathway, activation of D2 receptors inhibits striatal output neurons that project to the external globus pallidus (GPe). This results in the sequential inhibition of glutamatergic neurons in the subthalamic nucleus (STN) and GABA neurons in the GPi/SNr. Black arrows: indirect pathway; blue arrows: direct pathway.
(adapted from Conn *et al.*, 2005)

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submitted

Early life stress causes refractoriness to haloperidol-induced catalepsy

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Letter to the Editor

A common and serious drawback of classical antipsychotics is their association with pharmacological parkinsonism and other extrapyramidal motor syndromes (EPSs), which are a leading cause of noncompliance with medication (Casey and Keepers, 1988). Factors that predict the likelihood that classical antipsychotics cause (or not) EPSs are important for the design of a therapeutic regimen in patients with schizophrenia or bipolar disorders. The risk of developing pharmacological parkinsonism is influenced by age, gender, type and potency of antipsychotic drugs, genetic variants of drug-metabolizing enzymes, dose and duration of treatment, and history of previous antipsychotic-induced EPSs (Loebel *et al.*, 1992). Stressful events occurring early in life are established risk factors for schizophrenia, but how they influence the vulnerability to antipsychotic-induced parkinsonism is unknown. We examined haloperidol-induced catalepsy in "prenatal restraint stress (PRS) rats", i.e. the offspring of mothers exposed to repeated episodes of restraint stress during pregnancy (Maccari *et al.*, 1995).

Adult PRS rats and their unstressed controls were challenged with different doses of haloperidol (1, 2, or 5 mg/kg, i.p.) for the induction of catalepsy, which was monitored after 30, 60, 90, and 120 min. PRS caused a strong reduction of catalepsy at all times following injection of haloperidol at doses of 1 mg/kg (group effect, $F_{(1,8)}=17.54$ $p<0.01$) or 2 mg/kg (group effect, $F_{(1,8)}=16.13$ $p<0.01$). PRS rats were also resistant to catalepsy induced by high doses of haloperidol (5 mg/kg), but only in the first 60 min (Fig. 1A). Haloperidol levels in the serum and corpus striatum did not differ between control and PRS rats at 30 or 60 min after injection, although they were largely reduced at 120 min (serum: $t = 3.41$ $p<0.05$; striatum: $t = 4.43$ $p<0.01$) (Fig. 1B). Thus, changes in the pharmacokinetics of haloperidol could not fully explain the resistance of PRS rats to haloperidol-induced catalepsy. PRS rats were also hyper-responsive to the dopamine (DA) receptor agonist, apomorphine. Doses of apomorphine (0.3 mg/kg, s.c.) that normally produce only hyperlocomotion and sniffing caused oral diskynesias in PRS rats ($t = 2.56$, $p<0.05$) (Fig. 1C). This suggested the presence of neuroplastic changes in the basal ganglia motor circuit of PRS rats, which could alter responses to haloperidol or apomorphine. To identify these changes, we performed a stereologic counting of c-Fos-expressing neurons in the main stations of the basal ganglia motor circuit. GABAergic projection neurons of the neostriatum send signals to the primary output stations of the basal ganglia (i.e., the internal globus pallidus and the pars reticulata of the substantia nigra) both directly and indirectly through the external globus pallidus and the subthalamic nucleus. The "direct pathway" is positively modulated by D1 DA receptors, whereas the "indirect pathway" is negatively modulated by D2 DA receptors (reviewed by Conn *et al.*, 2005). Parkinsonism is associated with an increased activity of the indirect pathway and a reduced activity of the direct pathway, which both lead to an increased activity of the primary output stations. These, in turn, send inhibitory projections to ventral motor thalamic nuclei (VA, VL, VM), which project to the motor cortex. Parkinsonism is associated with a reduced activity of ventral thalamic nuclei (Conn *et al.*, 2005). In untreated PRS rats, the number of c-Fos$^+$ (active) neurons was unchanged in the external globus pallidus and subthalamic nucleus, reduced in the internal globus pallidus ($t = 3.05$, $p<0.05$), and almost doubled in VA, VL, and VM thalamic nuclei ($t = 5.37$; $p<0.01$) (Fig. 1D). This indicates a higher level of activity of excitatory thalamic neurons projecting to the cerebral cortex because the ventral motor thalamus is nearly devoid of inhibitory GABAergic interneurons (Sawyer *et al.*, 1991). Treatment with 2 mg/kg of haloperidol reduced the number of c-Fos$^+$ neurons in the VM thalamic nucleus after 1 hour in both control and PRS rats (treatment effect $F_{(1,12)} = 21.32$, $p<0.01$). However, c-Fos$^+$ neurons remained higher in PRS rats than in control rats after haloperidol ($p<0.05$), and the number of c-Fos$^+$ neurons in PRS rats treated with haloperidol did not differ from that measured in control rats treated with saline (Fig. 1E). These data suggest that an increased constitutive activity of ventral motor thalamic nuclei protects PRS rats against haloperidol-induced catalepsy.

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Haloperidol-induced catalepsy models pharmacological parkinsonism in humans and is highly sensitive to antiparkinsonian drugs, such as muscarinic cholinergic, A_{2A} adenosine receptor, and type-5 metabotropic glutamate receptor antagonists (Mandhane *et al.*, 1997; Ushijima *et al.*, 1997). Our data raise the interesting possibility that early life stress causes refractoriness to antipsychotic-drug induce parkinsonism in humans, and that the history of perinatal stress might influence the decision of using classical antipsychotics particularly in patients that are not eligible to treatment with atypical antipsychotics.

Conflict of interest

The authors declare no conflict of interest

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Figure legend

Fig.1. Early life stress causes reduction in haloperidol-induced catalepsy and increased neuronal activity in ventral motor thalamic nuclei.

Pregnant Sprague-Dawley female rats were subjected to three stress sessions daily (45 min. each) from day 11 of pregnancy until delivery according to our standard protocol (Maccari *et al.*, 1995). Only male offspring (3 month-old) from litters containing 10-14 pups with a comparable number of males and females were used for the experiments. Haloperidol-induced catalepsy is shown in (A). For measurements of catalepsy, the forepaws of each rats were placed on a horizontal bar (Nicoletti *et al.*, 1983). The immobility time was recorded for 300 sec at 30, 60, 90 and 120 min following systemic injection of haloperidol (1, 2 or 5 mg/kg, i.p.). Values are means ± S.E.M. of 5 determinations. *$p<0.05$ vs. the respective controls (Two-way ANOVA for repeated measures + Newman-Keuls). Levels of haloperidol in the serum and corpus striatum of control and PRS rats injected with 2 mg/kg of haloperidol are shown in (B). Levels were measured by LC-MS/MS (3,200 triple quadrupole system; Applied Biosystems, Foster City, CA). Values are means ± S.E.M. of 4 determinations. *$p<0.05$ (Student's t test) vs. the respective controls. Oral stereotypies in response to apomorphine are shown in (C). The number of episodes of liking, biting or gnawing was recorded during 30-min observation time starting 2 min following s.c. injection of apomorphine (0.3 mg/kg). Values are means ± S.E.M. of 8 determinations. *$p<0.05$ (Student's t test) vs. the respective controls. Stereologic counting of c-Fos$^+$ neurons in the external globus pallidus (GPe), subthalamic nucleus (STN), internal globus pallidus (GPi), and ventral motor thalamic nuclei (VA + VL + VM) of control and PRS rats is shown in (D). Values are means ± S.E.M: of 4 animals per group. *$p<0.05$ (Student's t test) vs. the respective control values. The number of c-Fos$^+$ neurons in control and PRS rats 1 hour following i.p. injection of saline or haloperidol (halo, 2 mg/kg) is shown in (E). Values are means ± S.E.M. of 4 rats per group. $p<0.05$ (Two-way ANOVA + Newman-Keuls) vs. the respective controls (unstressed) rats (*), or vs. the respective groups of rats treated with saline (#). Cell counting was carried out by the optical fractionator method using a Zeiss Axio Imager M1 microscope. The software Image-Pro Plus 6.2 (Media Cybernetics, Inc., Bethesda, MD) for Windows, equipped with a Macro (King *et al.*, 2000), was used for the analysis of digital images. The analysis was performed on 20 μm sections sampled every 240 μm. The total number of C-Fos$^+$ cells was computed using the following formula: $N = S(n) \times 1/SSF \times 1/ASF \times 1/TSF$, where n is the total number of cells counted on each dissector; SSF (fraction of sections sampled) the number of regularly spaced sections used for counts divided by the total number of sections across the area of interest; ASF (area sampling frequency) the dissector area divided by the area between dissectors; and TSF (thickness sampling frequency) the dissector thickness divided by the section thickness (17 mm/20 mm).

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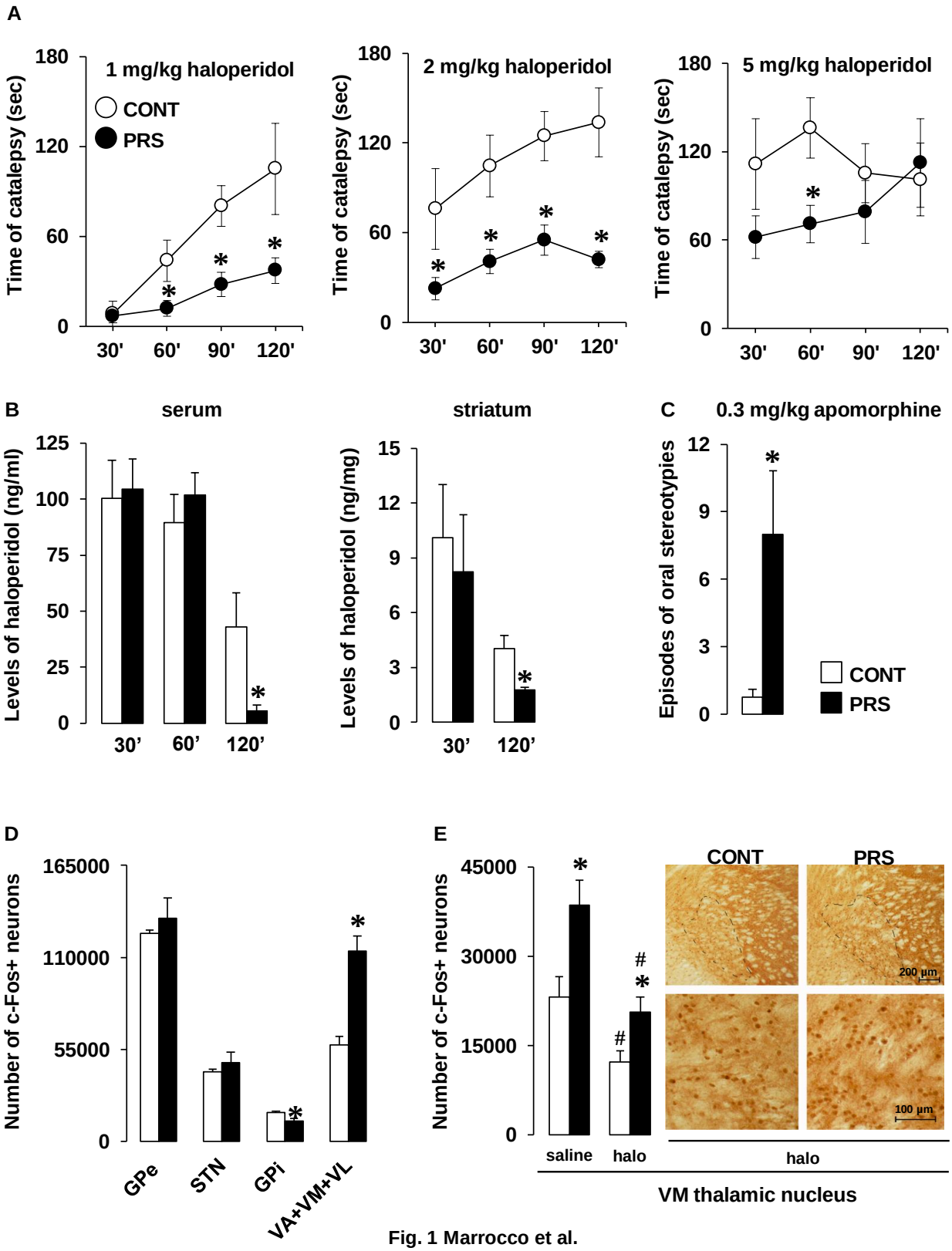

Fig. 1 Marrocco et al.

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GENERAL DISCUSSION

In the present thesis we have shown that hippocampal glutamatergic system was strongly impaired in the PRS model of depression in rat. These results fit nicely with findings that glutamate machinery is involved in the pathophysiology of depressive disorders (Sanacora *et al.*, 2012) and that hippocampus represents an integral part of the programming triggered by early life stress (Darnaudéry and Maccari, 2008).

a) PRS affects the hippocampal neuroproteome.

The hippocampal neuroproteome was deeply affected by PRS, and MS analysis revealed several proteins implicated in stress-related disorders such as lasp-1, fascin, reelin and transferrin. Interestingly, it has been shown that depressive patients exhibit low levels of transferrin and that this protein correlates negatively with patients' cortical-glutamatergic-mediated excitability (Salustri *et al.*, 2010). The construct validity of PRS as a model of depression was strengthen by data showing that the reduction in glutamate release was "corrected" by antidepressant treatment. Moreover, proteomics revealed that more than 20% of proteins affected by PRS, were involved in synaptic transmission and neural plasticity.

b) Chronic antidepressant treatment with agomelatine normalizes abnormalities of the hippocampal glutamatergic machinery.

Our data showed that the machinery of synaptic transmission was strongly impaired in the ventral hippocampus of PRS rats, and that, at least agomelatine, could reverse the pathological phenotype associated to reduction in both glutamate release and hippocampal synaptic vesicle proteins (SVP). It has been shown that SVP are involved in the pathophysiology of psychiatric disorders such as schizophrenic psychoses and depression (Eastwood *et al.*, 1995; Horner *et al.*, 1999; Vawter *et al.*, 2002). As a consequence, SVP result very sensitive to antidepressant treatment. Yamada and coworkers (2002) reported a differential expression of VAMP 2 in the frontal cortex of rats after antidepressant and electroconvulsive treatment whereas another study demonstrate that synaptophysin, synaptotagmin, synapsin I, and VAMP are all antidepressant-responsive genes (Rapp *et al.*, 2004). Thus, one can speculate that the beneficial action of antidepressants passes through the modulation of SVP. However, the direction of this modulation remains equivocal. Several works in control rats have shown that antidepressants aim to reduce SVP and glutamate release, thereby concluding that increasing in glutamate release was responsible of the depressed phenotype. Accordingly, it has been shown that chronic treatment with SSRI reduced the protein-protein interaction between syntaxin 1 and Thr286-phosphorylated αCaM kinase II (α-calcium/calmodulin-dependent protein kinase II). Data from the same group

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reported a marked increase in the interaction between syntaxin 1 and Munc-18 after antidepressant treatment (Bonanno *et al.*, 2005). Interestingly, SVP were found to be substrate for αCaM kinase II (Greengard, 1993; Popoli, 1993; Nielander *et al.*, 1995; Rubenstein *et al.*, 1993) and this kinase also mediated synaptotagmin phosphorylation after long term blockade of the specific-serotonin transporter SERT, the main target for SSRI (Popoli *et al.*, 1997). SERT can physically interacts with syntaxin 1A (Haase *et al.*, 2001) and the stoichiometry of this interaction is also regulated by αCaM kinase II (Ciccone *et al.*, 2008).

Chronic treatment with the antidepressant melatonergic receptor (MT(1)/MT(2)) agonist/5-HT2C receptor antagonist agomelatine was shown to abolish stress-induced increase of hippocampal synapsin I, indicating that non-classical antidepressants may also act on SVP modulation (Dagyte *et al.*, 2011). Nonetheless, PRS induced a reduction in the expression of SVP and glutamate release rather than an increase, thereby suggesting that the pathological phenotype could be programmed early in life to counteract the depressive-like phenotype at adulthood. In our study, only agomelatine was able to restore the levels of synapsin II, VAMP and Rab3a in PRS rats, while fluoxetine had no effects on the expression of these proteins. However, both fluoxetine and agomelatine increased glutamate release in the hippocampus of PRS rats and totally abolished the anxious/depressive-like behavior induced by PRS. This suggests two possible therapeutic pathways *via* the glutamatergic system where agomelatine acts through SVP-mediated glutamate release while fluoxetine-dependent glutamate restore seems not associated to SVP modulation.

These findings indicate a new line of antidepressant drug development that should be aimed at minimizing the effects of chronic stress exposure on the function of the glutamatergic neurotransmitter system (Sanacora *et al.*, 2008; 2012) (Fig. 17). Accordingly, it has been shown that drugs such as riluzole (Fumagalli *et al.*, 2008; Sung *et al.*, 2003; Rothstein *et al.*, 2005) and ceftriaxone (Mineur *et al.*, 2007), which increase glutamate clearance, can prevent or reverse the effects of chronic stress and chronic glucocorticoid exposure on amino acid neurotransmitter cycling, on glial expression within the prefrontal cortex, and on despair and anhedonia in animal models of depression (Banasr *et al.*, 2010; Gourley *et al.*, 2011). Also, a notable recent discovery shows that ketamine, a NMDA receptor antagonist, produces rapid (within hours) antidepressant responses in patients who are resistant to typical antidepressants (Duman and Aghajanian, 2012). (Fig. 18).

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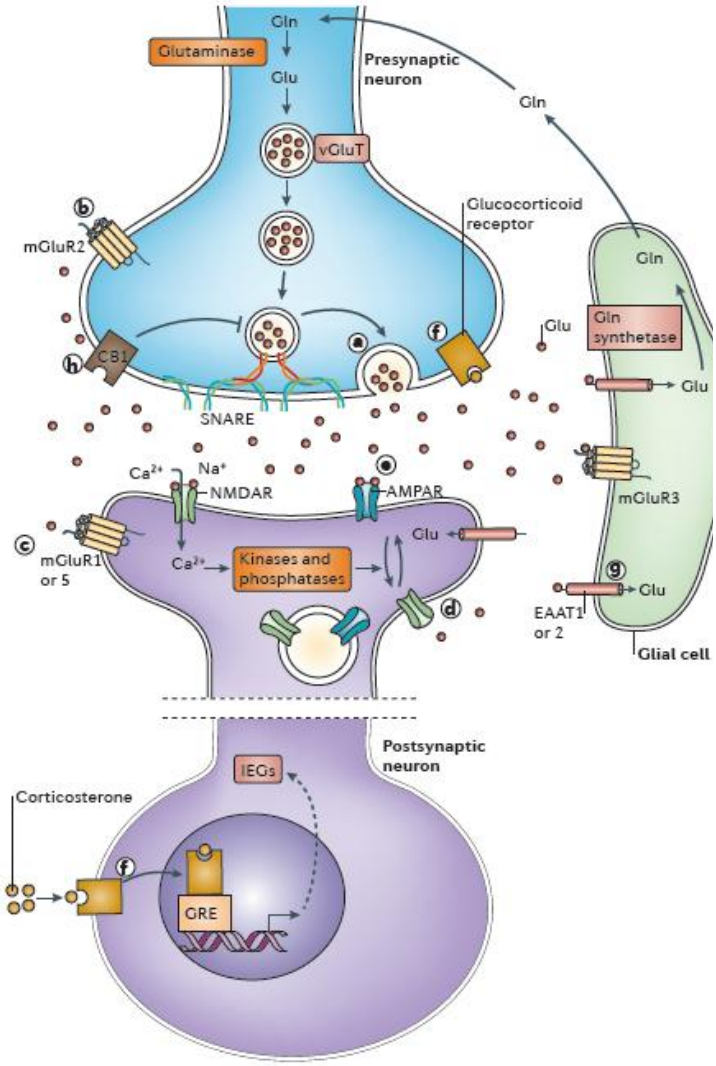

Figure 17. Pharmacological targets for antidepressant like-action. Drugs that modify glutamate release (**a**), such as lamotrigine and riluzole, antagonists. Negative allosteric modulators of the group II metabotropic receptors (mGluR2 and mGluR3) (**b**), such as MGS0039 and LY341495 and positive and negative allosteric modulators of mGluR5 (**c**). Drugs targeting NMDA receptors (**d**), and AMPA receptors (**e**). Agents that regulate glucocorticoid signalling (**f**). Drugs such as riluzole and ceftriaxone that indirectly facilitate glutamate transport into glia (**g**). Pharmacological augmentation of endocannabinoid signalling (**h**) EAAT, excitatory amino acid transporter; Gln, glutamine; GRE, glucocorticoid response element; IEGs, immediate early genes; vGluT, vesicular glutamate transporter (from Popoli *et al.*, 2012)

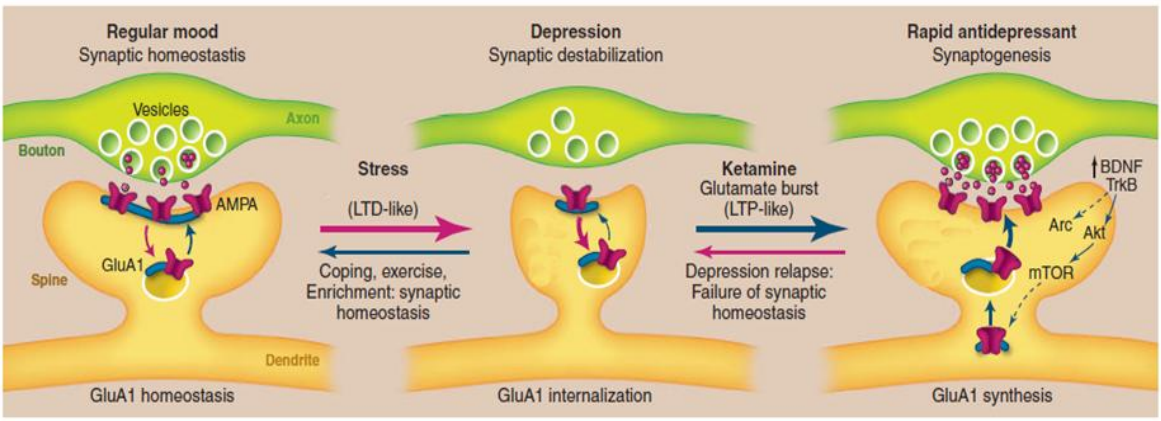

Figure 18. Model depicting the synaptogenic basis of depression and treatment response. (Left) Regular mood (synaptic homeostasis). Cycling of glutamate A1 (GluA1) receptors to and from the synapse levels of synapse number and function are maintained by homeostatic mechanisms. (Middle) Depression (synaptic destabilization). Chronic stress exposure decreases synaptic density, similar to LTD. (Right) Rapid antidepressants (synaptogenesis). Ketamine rapidly increases glutamate transmission and synaptogenesis, similar to LTP. Ketamine-induction of synaptogenesis requires BDNF/TrkB-activation of Akt and mTOR signaling, resulting in increased translation of synaptic proteins, which is required for the expansion and stabilization of spines (adapted from Duman and Aghajanian, 2012).

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c) Novel antidepressant/anxiolytic drugs with specific glutamatergic action.

Positive and negative allosteric modulators of metabotropic glutamate receptors, which can influence glutamate release and extracellular glutamate levels, have also been shown to have antidepressant-like actions (Krystal *et al.*, 2010) and are now being investigated for use in various psychiatric indications (Fig. 17). These strategies are strongly supported by the finding that neurotransmitters can even be released from non-synaptic varicosities without being coupled to frequency-coded neuronal activity and they are also able to diffuse over large distances (Vizi *et al.*, 2004), thereby including an extra-synaptic component for the modulation of classical synaptic transmission. For example, presynaptic mGlu2/3 receptors and $GABA_B$ receptors are known to negatively regulate glutamate release in the hippocampus and other brain regions (Chalifoux and Carter, 2011; Nicoletti *et al.*, 2011). This fits nicely with another interesting point of our study. We found, for the first time, that intrahippocampal injection of a cocktail containing presynaptic mGluR2/3 and $GABA_B$ receptor antagonists was able to both increase glutamate release in the hippocampus and reverse the anxiety-like behavior in PRS rats. Although we show no causal relationship between the cocktail-mediated glutamate release and SVP restoring, our results are likely consonant with recent data showing that a single dose of LY341495 was able to increase the level of synapsin I *via* the activation of the mammalian target of rapamycin (mTOR) (Dwyer *et al.*, 2012). Interestingly, in a preliminary neuroproteomic study, we detected mTOR among the proteins that were modulated by chronic treatment with agomelatine (data not shown). Also, Ago and coworkers (2012) have shown that LY341495 has an antidepressant-like effect on a mouse model of treatment-resistant depression. Surprisingly, our cocktail induced a region-specific increase in glutamate release, since the "enhanced release" was shown in ventral hippocampus but not in dorsal hippocampus.

d) The distinguishing role of ventral hippocampus:limits for translational pharmacology?

The reason why dorsal hippocampus was not sensitive to cocktail-induced increasing in glutamate release remains unclear. One can argue that mGlu 2/3 and $GABA_B$ receptors are differentially distributed in dorsal and ventral hippocampus, respectively, but this hypothesis warrants further investigations. Instead, Hörtnagl and coworkers (1991) found certain differences in the hippocampal dorso-ventral distribution of NA, 5-HT and GABA but not glutamate. Although the differential distribution of NA and 5-HT could be justified by the unequal number of inwards fibers within the hippocampus, glutamatergic fibers run within the dorso-ventral hippocampal formation and, therefore, no inwards glutamatergic fibers may have confounded our analysis. This raises a number of questions concerning the regional specific function of brain structures, especially hippocampus. Indeed, another key aspect of

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this thesis was to highlight the role of ventral hippocampus in emotion-related responses. We showed that PRS mainly affected ventral hippocampus both in glutamate release and SVP expression and that, at least glutamate release in the ventral region, was causally associated to the anxiety-like phenotype induced by PRS. Moving from these concerns, one can speculate a translational approach to the pharmacological treatment of mood disorders in humans. Yet, the actual translation of rodent data to humans, and thereby the understanding of the pathophysiology of neuropsychiatric disorders is limited (Berton *et al.*, 2012). The main reason for the translational flaw is that many behavioral tasks for rodents do not implement human task parameters (Holmes, 2003; Homberg, 2012). Furthermore, at least for cocktail-anxiolytic activity, we have suggested that "therapeutic" procedures should target specific brain regions, such as ventral hippocampus, whereas we have no knowledge on the action of the cocktail on behavior or glutamate release after a systemic treatment. Also, modern pharmacology advises against multiple pharmacological approaches in order to minimize drug side effects. However, the rational design of polypharmacology faces considerable challenges in the need for new methods to validate target combinations and optimize multiple structure-activity relationships while maintaining drug-like properties (Hopkins, 2008; Wang *et al.*, 2012). Thus, the clinical value of our findings should be examined by taking into account the aforementioned limitations.

e) *Is ventral hippocampus responsible of striatal response?*

Instead, we can argue an interesting translational hypothesis concerning our findings on the influence of PRS on striatal motor circuit. Indeed, haloperidol-induced catalepsy mimics pharmacological parkinsonism in human and therefore it represents an useful model to study striatal function. We found that PRS caused a strong refractoriness to haloperidol-induced catalepsy, as the result of an increased neuronal activity in the ventral motor thalamic nuclei. Haloperidol blocks dopamine D2 receptors and it is the most common classical antipsychotic drug, used to treat schizophrenia and schizophrenia-related disorders worldwide. Besides dopamine D2 receptor antagonism, that is a characteristic feature of all antipsychotic drugs, these agents also bind to a range of non-dopaminergic targets, including α-adrenergic and muscarinic receptors, serotonin, histamine, and glutamate (Nasrallah, 2008). Accordingly, it has been shown that glutamatergic gene variants impact the clinical profile of efficacy and side effects of classical antipsychotics, including haloperidol (Giegling *et al.*, 2011). These findings are consistent with the development of the glutamatergic theory of schizophrenia. Nowadays, glutamatergic models are based upon the observation that the psychotomimetic agents, such asphencyclidine (PCP) and ketamine, induce psychotic symptoms and neurocognitive disturbances similar to those of schizophrenia by blocking neurotransmission

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at NMDA-type glutamate receptors (Javitt, 2010). Furthermore, it has been shown that pretreatment with the non-competitive NMDA receptor antagonist MK-801 strongly attenuates haloperidol-induced c-Fos expression in the striatum (Ziolkowska and Hollt, 1993; Boegman and Vincent, 1996; Keefe and Adams, 1998; Hussain *et al.*, 2001) and that NR2B-NMDA subunit mainly contribute to this modulation (Lee and Rajacumar, 2003). Curiously, a number of results indicate that, in addition to NMDA receptors, also presynaptic proteins represent a potential molecular substrate for the effects of antipsychotics. It has been shown that haloperidol increases the expression of synapsin II both *in vitro* and in medial prefrontal cortex and nucleus accumbens (Chong et al 2006). Moreover, Barr and coworkers (2006) have shown that chronic treatment with haloperidol increased SNAP-25 throughout the tri-synaptic pathway of the hippocampus, with strongest effects in the mossy fiber region of CA3, suggesting that antipsychotic drugs can affect hippocampal synaptic connectivity and *vice-versa*. Indeed, one study has shown that neonatal ibotenic acid lesion of ventral hippocampus could both reduce haloperidol-induced catalepsy and increase apomorphine-induced hyperactivity (Lipska and Weinberger, 1993; Lipska *et al.*, 1995). Although this fits nicely with our results on PRS rats motor response to haloperidol and apomorphine, further investigations are needed to highlight a causal relationship between glutamatergic impairment in ventral hippocampus and striatal response to dopaminergic drugs. One can speculate that the unbalanced glutamatergic signal from ventral hippocampus to striatum may lead to abnormal response of group I mGlu receptors, that are widely expressed within the basal ganglia (Conn *et al.*, 2005; Nicoletti *et al.*, 2011).

Conclusive remarks

In conclusion, these results indicate that stress early in life may program the hippocampal glutamatergic system to cope with life events in adulthood. This lead to the development of an anxious/depressive phenotype that is sensitive to chronic treatment with antidepressants. Also, we showed that the glutamatergic system *per se* is highly responsive to antidepressant drugs, such as agomelatine and fluoxetine. We successfully demonstrated that both agomelatine and fluoxetine may exert their therapeutical action *via* the glutamatergic system. Also, we had for the first time the compelling evidence that a cocktail containing mGlu2/3 and $GABA_B$ receptors antagonists had a potent anxiolytic activity when injected locally in the ventral hippocampus.

Taken together these findings indicate that PRS is an attractive animal model to approach novel strategies for the treatment and the investigation of stress-related mood disorders.

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Perspectives

We attempted to prove that hippocampus integrates the functionality of related brain structures, such as striatum; yet, we have no evidence on the functional "inter-affection" of hippocampus and striatum in PRS rats. Instead, the finding that PRS are resistant to haloperidol-induced catalepsy, raises the possibility that anamnestic evaluation of perinatal stress might be important to predict whether or not a schizophrenic patient will develop pharmacological parkinsonism in response to classical antipsychotics, particularly when the individual is not eligible for treatment with atypical antipsychotics. Actually, most of atypical antipsychotics cause weight gain and metabolic syndrome, thereby increasing the risk for cerebrovascular disorders particularly in patients with Alzheimer's disease (Coccurello and Moles, 2010; Newcomer, 2005; Colton and Manderscheid, 2006; Leucht *et al.*, 2009; Lett, 2011). Remarkably, mGlu5 receptor antagonists but also antagonists of A_{2A} adenosine receptors are strongly effective in reducing haloperidol-induced catalepsy (Mandhane *et al.*, 1997; Ushijima *et al.*, 1997) and they are currently under investigation for the treatment of Parkinson's disease. In a parallel study that is not discussed in the present thesis, we found that PRS induced a pre-parkinsonian phenotype in adult rats. Indeed, PRS rats exhibited a marked reduction in striatal dopamine release and an increased expression and activity of striatal A_{2A} adenosine receptors (Bowalerh *et al.*, 2012). Thus, one may evince that ventral motor thalamic nuclei of PRS rats increased their neuronal activity to compensate a parkinsonian state upstream within the neostriatum or, *vice-versa*, that striatum works to counteract the aberrant activity of thalamus.

This analysis paves the way for the development of animal models of schizophrenia and Parkinson's disease and suggests that PRS, in rats, is also an excellent candidate to explore the pathophysiology of psychiatric and neurodegenerative disorders.

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Abstract

Stress is a major risk factor for mood disorders, such as anxiety and depression. Rats exposed to prenatal restraint stress (PRS) - i.e. the offspring of dams submitted to repeated episodes of stress during the last 10 days of gestation - develop long-lasting biochemical and behavioral changes that recapitulate some traits of depression and anxiety. Mounting evidence suggests the involvement of hippocampal glutamatergic system in such disorders. Interestingly, the hippocampus represents an integral part of the altered programming triggered by PRS. Hence, we decided to investigate the glutamatergic hypothesis of depression in the rat model of PRS focusing on mechanisms of neuroadaptation within the hippocampal circuit. We found that PRS rats showed an impairment of glutamate release, in the ventral hippocampus, which is the specific portion of the hippocampus related to stress and emotions. Remarkably, local injections of drugs that enhanced glutamate release in the ventral hippocampus (i.e., a cocktail of GABA-B and mGlu2/3 receptor antagonists) had strong anxiolytic effects in PRS rats. In addition, chronic treatment with conventional antidepressant drugs enhanced glutamate release in the ventral hippocampus and corrected the anxious/depressive-like phenotype induced by PRS. Knowing that the ventral hippocampus modulates striatal motor programming, we extended the study of PRS rats to haloperidol-induced catalepsy, which models pharmacological parkinsonism in humans. We found that PRS rats were resistant to haloperidol-induced catalepsy as a result of an increased activity of motor thalamic nuclei, as assessed by stereologic counting of c-Fos-positive neurons. Our findings support the glutamatergic theory of stress-related mood disorders and suggest that an impairment of the ventral hippocampus and its influence on striatal circuit are key components of the neuroplastic program induced by PRS.

Résumé

Le stress est un facteur de risque majeur pour les troubles de l'humeur comme l'anxiété et la dépression. Les rats exposés à un stress prénatal de contention (PRS) – *i.e.* la progéniture de mères soumises à des épisodes répétés de stress au cours des 10 derniers jours de gestation-développent des changements biochimiques et comportementaux durables qui résument certains traits de la dépression et de l'anxiété. Un nombre grandissant de travaux suggère l'implication du système glutamatergique hippocampique dans ces troubles. L'hippocampe fait partie intégrante de la programmation altérée déclenchée par le PRS. Nous avons donc décidé d'étudier l'hypothèse glutamatergique de la dépression chez le rat PRS, en mettant l'accent sur les mécanismes de neuroadaptation dans le circuit hippocampique. Nous avons démontré que les rats PRS présentaient une altération de la libération de glutamate dans l'hippocampe ventral, partie spécifique de l'hippocampe reliée au stress et aux émotions. Remarquablement, des injections locales de produits qui améliorent la libération du glutamate dans l'hippocampe ventral (*i.e.* un cocktail d'antagonistes des récepteurs au GABA-B et mGlu2/3) exerçaient un fort effet anxiolytique chez les rats PRS. De plus, un traitement chronique avec des antidépresseurs conventionnels améliorait la libération du glutamate dans l'hippocampe ventral et corrigeait le phénotype de type anxieux/dépressif induit par le PRS. Sachant que l'hippocampe ventral module la programmation motrice striatale, nous avons étendu notre étude chez le rat PRS à la catalepsie induite par l'halopéridol, qui modélise le parkinsonisme pharmacologique chez l'Homme. Nous avons constaté que les rats PRS étaient résistants à la catalepsie induite par l'halopéridol, comme le résultat d'une activité augmentée des noyaux thalamiques moteurs, et tel que révélé par le compte stéréologique des neurones c-Fos-positifs. Nos résultats renforcent la théorie glutamatergique dans les troubles de l'humeur liés au stress et suggèrent qu'une déficience de l'hippocampe ventral et son influence sur le circuit striatal sont des éléments clé du programme neuroplastique induit par le PRS.

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