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PRENATAL STRESS IN RAT, AN ANIMAL MODEL OF DEPRESSION: BRAIN PLASTICITY, CIRCADIAN DISORDERS AND NEW ANTIDEPRESSANTS.

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A chi ha asciugato le mie lacrime che troppe volte hanno rigato il mio viso,

a chi mi ha insegnato che per raggiungere la meta bisogna esser tenaci,

a chi, come me, crede che l'isola che non c'è non è solo un'invenzione,

a chi mi ha sempre detto che basta crederci per trovare la strada,

a chi nei momenti bui, che non sono mancati, mi ha sorretto,

a chi riesce a strapparmi un sorriso nei momenti più tristi,

a chi mi ha indicato la strada e mi ha aituto a crescere,

a chi mi ha sempre sostenuto e continuerà a sostenermi,

a chi mi ha sopportato e, spero, continuerà a farlo,

a chi mi è sempre vicino e a chi ormai non c'è più,

a chi ogni giorno vive la vita insieme con me,

a chi non c'è ancora, ma presto ci sarà,

a chi con pazienza mi ha ascoltato,

a chi è la ragione della mia vita,

a chi rende realtà i miei sogni,

a tutti quelli che amo,

a chi mi ama,

a VOI,

a ME…

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ENGLISH ABSTRACT

BACKGROUND It is recognized that exposure to an adverse environment during foetal period may have lifelong programming effects on different body functions with a considerable impact on disease susceptibility. The stressors occurring during pregnancy can impair biological and behavioural response to stress during adulthood. Prenatal restraint stress (PRS) in rat is a well-documented model of early stress known to induce long-term neurobiological and behavioural alterations and it is a validated model to study anxiety and depression like-behaviours.

AIMS This work tries both to better characterise the phenotype of PRS rats in regard to circadian rhythms (locomotor activity and sleep/wake cycle) and hippocampal neuroplasticity and to test the capability of two antidepressants to reverse the alterations induced by PRS.

RESULTS Significant phase advances in circadian rhythms of locomotor activity were observed in PRS rats compared to controls, also after an abrupt shift of L/D cycle. The sleep/wake cycle of PRS was significantly more erratic and fragmented compared to controls. Brain plasticity (hippocampal neurogenesis, mGlu receptors expression, protein expression) was reduced by PRS. However antidepressants treatment was able to reverse the PRS abnormalities and to back to the level of the control group the parameters considered.

CONCLUSIONS Those observations both reinforce the idea of a general homeostatic dysfunction in animals exposed to prenatal stressful events that might partially explain some of their abnormal hormonal/behavioural response to stress and could increase the comprehension of the mechanisms underlying the long-term effects of early life manipulations.

FRENCH ABSTRACT

CONTEXTE: Le développement d'un individu se fait sous l'influence de son patrimoine génétique et de facteurs épigénétiques. Ainsi des perturbations environnementales précoces telles qu'une exposition accrue aux glucocorticoïdes maternels pourraient favoriser et programmer l'émergence de désordres physiologiques et comportementaux à l'age adulte. Chez le rat, un stress prénatal (PRS, stress de contention chronique chez la mère gestante) modifie de manière permanente l'activité de l'axe corticotrope de la descendance, il induit, également, une réduction de la neuroplasticité tout au long de la vie de l'animal ainsi que des perturbations durables sur le plan comportemental. Celles ci seraient associées à des perturbations neurologiques et endocrines similaires à celles observées au cours de la dépression chez l'Homme.

BUT DE LA THESE etait de caracteriser le phenotype des rats PRS au niveau des ryhtmes circadiens (activité locomotrice et cycle veille-sommeil) et la neuroplasticité hippocampique ainsi que d'évaluer la modulation pharmacologique des altérations induites par le PRS à travers deux types d'antidepresseurs.

RESULTATS Les rats PRS montrent une avance de phase du rythme circadien d'activité locomotrice ainsi qu'une fragmentation importante du cycle veille sommeil par rapport aux animaux contrôles. De façon générale le PRS induit une réduction de la neurogénèse hippocampique et de la neuroplasticité. Nous avons pu verifier la validité predictive du modèle PRS dans sa réponse aux antidépresseurs. Certaines des alterations comportementales ainsi que toutes les alterations de la plasticité cerebrale que nous avons observées peuvent être réduites par differentes classes d'antidepresseurs.

CONCLUSION Ces résultats font du modèle de stress prénatal de contention un modèle intéressant de programmation précoce des maladies de l'adulte pour étudier la relation entre un dysfonctionnement de l'axe corticotrope et certaines altérations comportementales et neurologiques.

FRENCH SUMMARY

La fonction et le dysfonctionnement de cerveau tout au long de la vie sont déterminés par l'interaction des facteurs génétiques avec des événements, des signaux et des stimuli environnementaux « acquis » (McEwen, 1999). Les facteurs génétiques contribuent de façon cruciale à la fonction du cerveau, tandis que les événements qui se produisent tôt dans la vie sont capables d'exercer des effets qui persistent tout au long de l'âge adulte, comme au niveau de la NEUROPLASTICITE; l'exposition au stress, au cours de la période critique du développement, occupe une place prépondérante parmi ces événements qui exercent une influence durable sur la fonction de cerveau.

Le stress déclenche une cascade d'évènements moléculaires qui entraine des réponses comportementales, autonomes et cognitives rapides du CNS aux circonstances de stress, suivies d'un retour rapide à l'état d'équilibre sur le plan fonctionnel. Dans ce mécanisme on observe non seulement la sécrétion rapide des molécules effectrices, mais également, un changement prolongé et coordonné de l'expression programmée de certains gènes (Avishai-Eliner et al., 2002).

Pour nos travaux, nous avons utilisé un modèle de stress de contention prénatal (PRS, Prenatal Restraint Stress). Chez le rat, ce modèle de stress prénatal précoce présente des altérations à long terme des fonctions neuronales et du développement, des désordres biochimiques, endocrinologiques et comportementaux. L'impact du PRS étant déjà décelable chez le foetus, il est évident qu'une programmation précoce par le stress prénatal induit des pathologies physiologiques chez l'adulte. Les études menées à ce jour par notre équipe et par d'autres ont permis de mettre en évidence l'aspect et le caractère prédictif de ce modèle puisque des anomalies observées chez le rat PRS sont comparables, dans une certaine mesure, à celles trouvées dans la dépression humaine (Maccari et al.,

2003; Maccari et Morley-Fletcher, 2007). Il s'agit de symptômes neurobiologiques de la dépression et non du syndrome de la dépression comme les humeurs qui ne peuvent pas être reproduits chez le modèle animal. En fait, il est tout à fait évident que l'étiologie d'une maladie psychiatrique complexe comme la dépression chez l'homme ne puisse pas être réduite simplement à quelques changements biologiques montrés chez le rat. Cependant, ce modèle animal nous permet certes de mieux comprendre les mécanismes d'action des antidépresseurs sur des symptômes spécifiques mais également de développer des drogues plus appropriées et ciblées, augmentant leur efficacité dans le traitement de la dépression. Il est d'ailleurs intéressant de noter que les anomalies spécifiques observées chez le rat PRS peuvent être efficacement contrecarrées par des traitements chroniques par des antidépresseurs.

Objectifs

Nos études neurobiologiques ont été menées sur l'HIPPOCAMPE, région clé impliquée dans la régulation de la réponse au stress et la neuroplasticité. Elle constitue une cible « prometteuse » de l'action des antidépresseurs.

a) La première série de travaux de cette thèse de PhD était de mieux caractériser le PHÉNOTYPE des rats PRS, en étudiant à la fois leurs états comportementaux et neurochimiques. Cette caractérisation permet de découvrir de nouveaux caractères comparables à ce qui est observé dans la dépression et de développer de nouvelles thérapies.

Pour cette caractérisation, notre objectif a été de répondre aux questions suivantes :

1) le PRS influence-t' il les **rythmes circadiens**, en particulier l'activité locomotrice, le cycle sommeil/activité et l'expression journalière de CRH?

Différentes études récentes ont confirmé que les altérations du sommeil et de l'activité locomotrice entrainent, de façon importante, une diminution de la neurogénèse hippocampique ce qui pourrait initier la dépression.

2) le PRS a-t-il des conséquences sur la **neurogénèse hippocampique** et sur l'expression de certaines protéines neuronales?

Deux raisons principales nous ont amenés à étudier la neurogénèse : tout d'abord, le stress constitue un des inhibiteurs les plus efficaces de cette neurogénèse; les stresses qui sont prolongés ou extrêmes, peuvent avoir comme conséquences des changements anormaux de la plasticité du cerveau; en second point, différentes expériences menées ces dernières années suggèrent qu'un changement de plasticité du cerveau, en particulier au niveau de la neurogénèse hippocampique pourrait être la cause de la dégénération neurobiologique typique rencontrée dans la dépression.

3) le PRS a-t-il des effets sur l'ontogenèse des **récepteurs métabotropiques au glutamate**?

Notre étude a porté sur les récepteurs métabotropiques impliqués dans la plasticité neuronale et synaptique. En effet, de nombreuses données expérimentales, comportementales et biochimiques, ont permis de mettre en évidence que la régulation de la neurotransmission glutamatergique via les récepteurs mGlu est corrélée aux désordres de l'humeur et du sommeil. Par ailleurs, ces récepteurs peuvent constituer de nouvelles cibles pour la découverte de petites molécules modulatrices possédant des propriétés uniques de type antidépresseur. Par exemple, la modulation des récepteurs mGlu peut réguler la neurogenèse et la libération de neurotransmetteurs qui sont impliqués dans la réponse aux traitements de la dépression chez l'Homme.

b) Notre seconde série de travaux était de confirmer la valeur prédictive du modèle de PRS et d'étudier les antidépresseurs les plus appropriés et les plus efficaces pour annuler les symptômes spécifiques de la dépression. Nous avons analysé la capacité de nouveaux antidépresseurs, **AG0310**, et **fluoxetine** (Prozac®) à annuler les modifications des rythmes circadiens et de la plasticité de cerveau induites par le PRS. Nous avons été amenés à répondre aux questions suivantes :

1) l'AG0310 est-il capable de «renverser» les modifications induites lors d'un PRS sur les rythmes circadiens veille/sommeil et l'activité locomotrice?

Nous avons déterminé si l'AG0310 peut normaliser les rythmes circadiens préalablement modifiés par le stress prénatal, améliorant ainsi les rem du sommeil et l'activité locomotrice. En effet, une désynchronisation de rythme biologique chez des patients déprimés comme le dysfonctionnement du sommeil est associé à l'hyperactivité d'axe hypothalamo-hpophyso-surrénalien (HPA, Hypotalamus-Pituitary-Adrenal). L'AG0310 est décrit comme un agoniste des récepteurs mélatoninergiques de type $MT_{(1)}$ et $MT_{(2)}$, et comme un antagoniste des récepteurs à la sérotonine, $5\text{-}HT_{(2C)}$. Son utilisation constitue une approche pharmacologique innovatrice dans le traitement de la dépression, si l'on considère aussi que l'AG0310 présente une bonne efficacité et que la molécule est généralement bien tolérée.

2) la fluoxetine est-elle capable de corriger les anomalies provoquées par le PRS sur la neurogenèse hippocampique?

Nous nous sommes intéressés à la capacité de la fluoxetine d'annuler la diminution de la neurogenèse hippocampique induite par le PRS chez des rats : plusieurs antidépresseurs sont connus comme permettant l'augmentation de la prolifération et de la neurogénèse des cellules du gyrus denté de l'hippocampe chez l'adulte.

L'ensemble de nos résultats présenté dans la première partie de ce mémoire a permis de mettre en évidence que le PRS entraine des modifications importantes de la neuroplasticité du cerveau et des anomalies des fonctions circadiennes et du sommeil. En fait, des avances de phase significatives des rythmes circadiens de l'activité locomotrice chez des rats PRS sont observées ce qui n'est pas le cas chez les animaux contrôles. On observe également chez les animaux PRS un décalage de phase brutal du cycle jour/nuit. Le cycle de sommeil/activité chez des rats PRS est sensiblement plus erratique et fragmenté par rapport aux animaux contrôle. Par contre, la plasticité cérébrale que ce soit au niveau de la neurogenèse hippocampique, de l'expression des récepteurs au mGlu, de l'expression protéique, est réduite chez les rats PRS. Ainsi, nos résultats suggèrent que l'environnement prénatal exerce des influences profondes sur le développement de l'organisme, induisant des changements qui commencent de façon précoce et se prolongent assez tardivement au cours de la vie.

L'intérêt des altérations des rythmes circadiens après un stress prénatal chez la progéniture adulte, réside dans le fait que le sommeil est nécessaire pour la survie de l'individu. Il s'agit d'un événement physiologique d'importance capitale, puisque, pendant le sommeil, la récupération des fonctions fondamentales de l'organisme a lieu et les processus de mémorisation sont consolidés. Par ailleurs, les modifications du sommeil induites par le PRS sont bien liées à la diminution du neurogénèse de l'hippocampe comme nous avons pu le montrer, puisqu'elle est réduite par la fragmentation du sommeil chez le rat adulte.

Les modifications comportementales chez les rats PRS, que nous avons pu décrire au niveau des rythmes circadiens, de la diminution de l'activité de l'axe HPA et de la perturbation dans la plasticité hippocampique, peuvent être comparées, dans une certaine mesure, à certains troubles décrits chez une grande majorité de patients

déprimés. Ces résultats suggèrent que le PRS constitue un modèle d'étude chez l'animal pour étudier les symptômes de la dépression. Par ailleurs, contrairement à d'autres modèles animaux de dépression, le fait que les anomalies induites par le PRS persistent, présente un intérêt fondamental pour concevoir et tester des stratégies thérapeutiques des désordres dépressifs.

Nos résultats présentés dans la seconde partie de ce mémoire de thèse démontrent que les antidépresseurs que nous avons étudiés, sont capables d'annuler les changements induits par le PRS et de retrouver des paramètres physiologiques à un niveau comparable à ceux observés chez les animaux contrôles. Ces données renforcent la validité prédictive du modèle de PRS.

FOREWORD: STRESS, CONCEPT AND DEFINITION

Concepts of stress and homeostasis

An organism life requires the incessant execution of adaptation processes to environmental variations. The life exists through the maintenance of a complex dynamic equilibrium of the internal environment called "**homeostasis**", which constitutes one constant challenge to the intrinsic or extrinsic unfavorable forces, real or perceived: the stressors (Habib et al., 2001). "Le milieu intérieur" is the original concept of Claude Bernard (1868) according to whom the internal environment is maintained in a constant balance even if the conditions of the environment change. Specifying this concept, Cannon proposed in 1932 the term of **homeostasis**. This term results from the Greek *homo* (same, like) and *stasis* (to stand, posture). Cannon was the first to study the variations of the physiological response to threatening environmental conditions (Cannon, 1929). He ground his proposal on the idea according to which stable states like the level of glucose, the body temperature and acido-basic balance are closely controlled. This stability requires that any tendency to change meets automatically factors of resistance. When environmental changes appear important or unforeseeable the mechanisms of the stress responses are activated. These responses require the intervention of the whole, central and peripheral nervous system, involving neuroendocrine and immune responses which activate adaptive functions of survival and, later, ensure the return to the equilibrium of the homeostatic patterns. Within this framework, the stress is defined as situation threatening or which is perceived like a threat to the homeostasis. The term of stress was first defined by Hans Selye in 1935.

Everyone has personal understanding of stress and to find a definition is difficult. Stress is a "multidimensional concept" built around at least three components:

- the stimulus, or stressor, can be positive or negative;

- the cognitive evaluation of the stressor, which depends on the previous life experience of the individual and on his/her ability to predict the stressful experience;

- the resulting physiological response(s) of the individual.

This third component refers to Selye's characterization of the stress response as a "general adaptation syndrome", organized into three stages (Selye, 1976). The first stage is the general alarm reaction, during which numerous biological systems (including the neuroendocrine axis) are activated in response to the stressor. The second stage would lead to resistance. If the stressful stimulus is maintained, the organism loses its resistance and enters in a phase of exhaustion, regarded as the third stage of the syndrome.

The consequences of physiological activation are many: the energy is mobilized (such as free fatty acids, glycerol, glucose, amino acids) from the storaged nutrients (triglycerides, glycogen, proteins) and when the energy storage ceases, the cardiovascular/pulmonary tone augments and the delivery of oxygen and glucose to tissue is facilitated, and thus, anabolic processes, suppression of digestion, growth, reproduction, inflammatory responses and immunity slow down until the acute emergency has passed (Sapolsky, 1992).

Cognition is simultaneously altered, with a tendency towards sharpened sensory thresholds, logical adaptation for coping with an emergency situation. At the same time, negative feedback mechanisms are activated to counteract the physiological activation and reinstate a new equilibrium. If these feedback mechanisms succeed, the organism will be able to deal with the stressful situation, eliminate its source and initiate appropriate behaviours: stress is, then, an adaptive response which enables the organism to cope with daily threatening environmental stimuli. If the sources of stress are prolonged and/or uncontrollable, feedback mechanisms fail in restoring

the equilibrium; then, the stress response becomes inadequate and may ultimately result in various pathological states (e.g. hypertension, cardiomyopathy, G.I. ulcerations) including sleep and mood disorders (Van Reeth et al., 2000).

Allostasis, the allostatic overload and cerebral plasticity

Currently, it is possible to distinguish **homeostasis** ("constancy in stability") from an alternative model of regulation called "**allostasis**" ("stability or homeostasis in the change"). The allostasis means phenomena of adaptation which maintain homeostasis with the production of mediators like the glucocorticoids, adrenaline, and other chemical messengers. These mediators of the stress response support the adaptation in the wake of the acute stress, but they can also contribute to the allostatic overload when the fact of "being stressed" wears off the body and the mind following a prolonged release of these mediators. The concept of **allostasis** suggests that the goal of the regulation is not constancy (Sterling and Eyer, 1988) and replaces in the centre of the adaptive processes the **plasticity** of the brain structures that control the release of the stress mediators. The **cerebral plasticity** can be defined as the ability of the brain to change, both its activity and its morphology (synapses, number of cells…) in response to environmental changes (Zilles, 1992). The deterioration of the organism abilities to cope adequately with the stressors, as for example by producing excessive or prolonged responses, can lead to the "**allostatic load,** then, to an **overload**" (McEwen, 1998), thus, conducing to the development of metabolic as well as cerebral pathologies. The "**allostatic overload**" consists in a reduction of the plasticity of the biological systems. This "allostatic overload" is, often, associated to the appearance of cardiovascular diseases like arterial hypertension, metabolic disorders like the diabetes insulino-resistant or obesity, psychiatric like the depression (McEwen, 2005).

Do adverse early life events predispose to an allostatic overload?

Although many individuals experiencing stressful events do not develop such pathologies, stress seems to be a provoking factor in individuals with particular vulnerability, determined by genetic factors or **early life events** (McEwen and Sapolsky, 1995). **Prenatal restraint stress (PRS)** in the rat induces a hyperactivity of the hypothalamo-pituitary-adrenal (HPA) axis in response to the stress in the adulthood associated with an increase of the anxious and depressive-like behaviours (Maccari et al., 2003). Thus, it seems that the PRS predisposes to emergence of an allostatic overload and/or deteriorates the processes of cerebral plasticity. However, it is unknown how these deteriorations of the allostasis processes could be associated with impairments of cerebral plasticity.

The models of early stress in rodents, like prenatal restraint stress in the rat, are often presented like models reproducing some of the features of human psychopathology such as the depression. The use of these models in this field of application remains for as much discussed and debatable. Indeed, the biomedical research is based for a long time on the use of animal models in the study of the biological bases of a large variety of diseases, the cerebral and behavioural disorder not making exception. However, the use of the animal models in the research field of psychopathologies such as depression is risky because are performed studies in nonhuman animals of disorder defined by cognitive and emotional processes, typically human (ex: mood depressed or self regard reduction in the major depression). It remains possible to use animal models and in particular rodents for the study of the cognition and behavioural disturbance, but some precautions are required:

- the assumptions on the psychological processes which cannot be measured in the rodent must be eliminated from the field of investigation;

- it is by far preferable to focus the study on the symptoms, instead, than on the syndrome. Rather than to observe the human syndrome overall and to compare it with the disorder presented by the animal model by making them correspond to a common aetiology, it could be more judicious to analyse individually each symptom and its origin;

- a neurobehavioral mechanistic approach, in which neurobiological hypothesis rather than psychological hypothesis are used as mechanisms for discrete symptom, will yield more useful information regarding the nature and treatment of the depression (for review: Holmes, 2003).

Other than the previous precautions, it is essential a detailed knowledge of the biological systems altered in the depression and anxiety disorders before to approach the animal modeling of the symptoms of these phatologies.

FIG. 1 – Schematic representation of a multidimensional concept of stress around three important concepts: the stimulusstressor, its cognitive evaluation by the organism and resulting physiological response(s). The activation of various biological systems in relation to brain plasticity and the allostatic process (including neuroendocrine and behavioral responses) leads the organism to restore its equilibrium, resulting in the activated biological systems, return to normal but not necessarily to the same equilibriumas before the stimulus. If the source(s) of stress is prolonged and/or uncontrollable, feedback mechanisms fail in restoring equilibrium, the stress response becomes inadequate and may ultimately result in various pathological states, including sleep and mood disorders. This incapacity to restore equilibrium increases vulnerability to disease. Epigenetic factors could play an important role. From Darnaudéry and Maccari, 2007

INTRODUCTION

1. EARLY LIFE EVENTS AND THE FOETAL PROGRAMMING

1.1 Foetal programming

During the past decade, a considerable body of evidence has emerged showing that circumstances during the foetal period may have lifelong programming effects on different body functions with a considerable impact on disease susceptibility.

The concept of early life physiological "programming" or "imprinting" has been advanced to explain the associations between prenatal environmental events, altered foetal growth and development, and later pathophysiology (Csaba, 1986; Seckl, 1998). Programming reflects the action of a stimulus or insult, during a sensitive developmental period or "window", to affect the development and organization of specific tissues that are concurrently vulnerable.

Of course, different cells and tissues are sensitive at different times, so the effects of environmental challenges will have distinct effects that depend not only on the challenge involved, but, also, on its timing. In other words, one genotype may give rise to different phenotypes based on conditions during early development, which is referred to as "developmental plasticity" (Bateson et al., 2004). In evolutionary terms, such plasticity during development may be advantageous in adjusting the metabolic needs or behaviour of an individual to environmental conditions that are likely to prevail during the life-course (Kajantie, 2008).

Examples of prenatal manipulation are prenatal stress, exposure to synthetic glucocorticoids and nutrient restriction. Postnatal manipulations that provoke alterations in adult offspring include neonatal handling, maternal deprivation, modified maternal behaviour, exposure to synthetic glucocorticoids and infection.

Although foetal responses to such exposures may impart an enhanced capacity to cope with the immediate stressor, such in utero "developmental programming" can contribute a significant risk for a number of important adult diseases. For instance, an adaptation to an intrauterine environment in which oxygen and nutrients may be limited has been suggested to promote the development of a "thrifty phenotype", reducing foetal growth and favouring metabolic efficiency (Hales and Barker, 1992). The long-term effects of early-life conditions include cardiovascular disease, type 2 diabetes, cognitive impairment, depression and osteoporosis.

Previous studies conducted to determine the role of maternal glucocorticoids in the programming of the HPA function of the adult convincingly indicate that increased transfer of glucocorticoids from the mother to the foetus is the key factor in this prenatal imprinting (Barbazanges et al., 1996; Seckl, 2000). However, further studies are necessary to determine the mechanism by which maternal glucocorticoids program adult offspring behaviour.
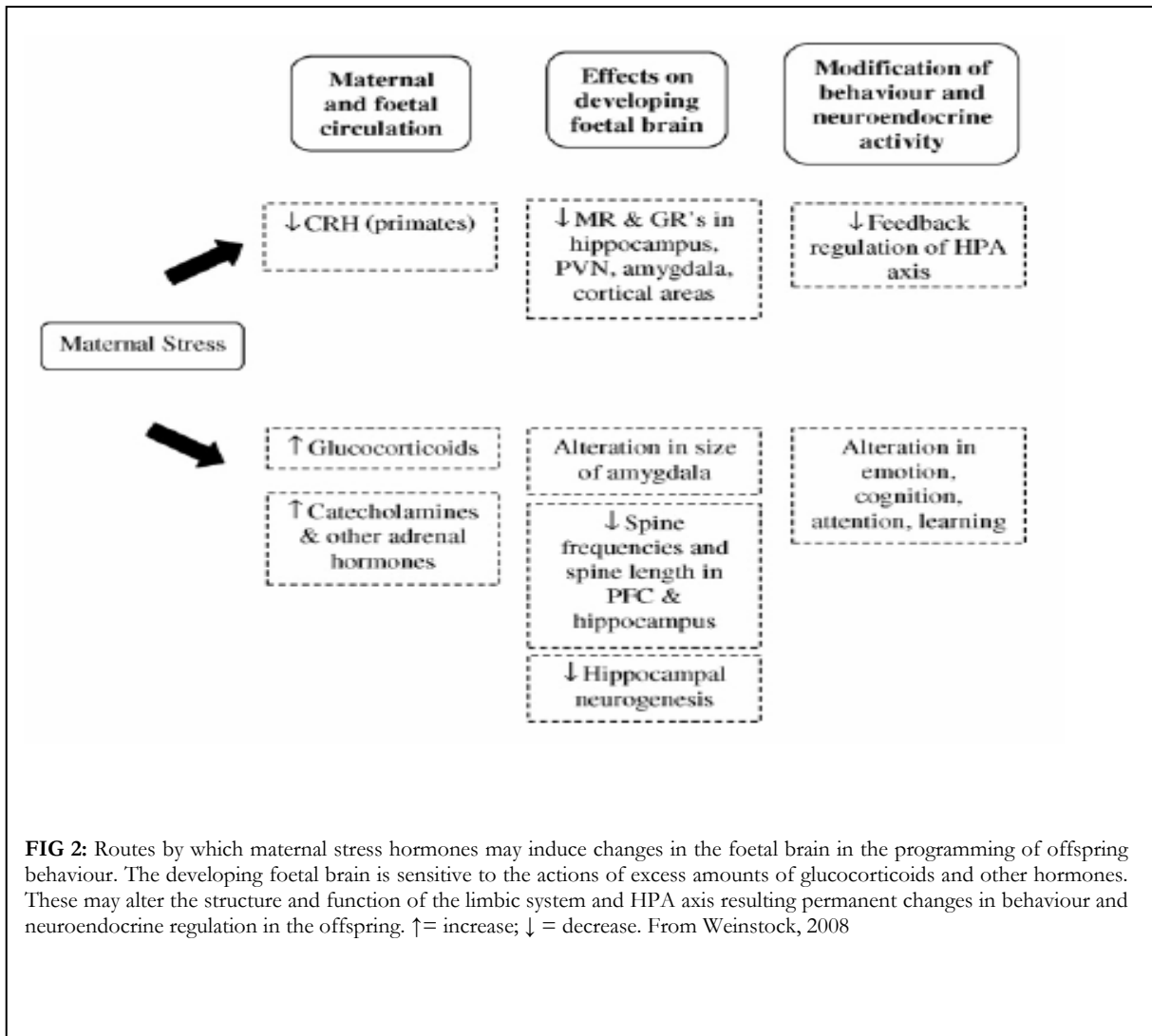
FIG 2: Routes by which maternal stress hormones may induce changes in the foetal brain in the programming of offspring behaviour. The developing foetal brain is sensitive to the actions of excess amounts of glucocorticoids and other hormones. These may alter the structure and function of the limbic system and HPA axis resulting permanent changes in behaviour and neuroendocrine regulation in the offspring. ↑= increase; ↓ = decrease. From Weinstock, 2008

1.2 Role of glucocorticoids and molecular mediators

Glucocorticoids, steroid hormones produced predominantly by the adrenal gland, are key mediators of stress responses. Whilst the acute and chronic effects of pharmacological glucocorticoid excess are well-recognised, their role in the biology of the response to stress is more nuanced, with balanced homeostatic effects to facilitate short-term survival and recovery from challenge (Munck and Naray-Fejes-Toth, 1994; McEwen, 2007). In addition, glucocorticoids are crucial during foetal development for the maturation of tissues and organs, promoting cellular differentiation, and most notably acting during late gestation to stimulate surfactant

production by the lung. This action is critical to prepare the foetus for extrauterin life, and it is for this reason that synthetic glucocorticoid treatment is so widely used in preterm pregnancies where lung immaturity threatens neonatal viability. Although these treatments greatly improve survival (Roberts and Dalziel, 2006), they are not without adverse affects.

Numerous studies across a wide range of species have shown that prenatal treatment with glucocorticoids reduces birth weight and that these offspring are at increased risk of cardio-metabolic disease, HPA axis perturbations and affective disorders in later life (Seckl, 2004). Moreover, in human pregnancies complicated by intrauterine growth restriction (IUGR), foetal cortisol levels are elevated at term (Goland et al., 1993), associating reduced foetal growth rates with elevated glucocorticoids. The effects of exogenous glucocorticoids on birth weight are greatest when administered during periods of rapid growth, typically the later stages of pregnancy (Nyirenda et al., 1998). It has been shown in rats that excess maternal glucocorticoids are the key in mediating the effects of maternal stress or diet on offspring hypertension (Langley-Evans, 1997; Lesage et al., 2001). Inhibition of maternal glucocorticoid synthesis using metyrapone in pregnant rats prevents the increased blood pressure seen in offspring of low protein diet fed rat dams. Replacement of corticosterone to the dam reinstated the programming effects, interestingly only in female offspring (Langley-Evans, 1997). Maternal adrenalectomy likewise prevented the reduction in foetal adrenal weights seen with maternal food restriction; however, foetal body weights were still lower in adrenalectomised and food-restricted foetuses (Lesage et al., 2001).

These studies lend support to the idea that maternal glucocorticoids mediate at least a component of the programming effects of foetal development on offspring HPA axis perturbations. A number of the adult diseases associated with the "low birth weight baby syndrome" involve perturbations in key glucocorticoid responsive

tissues, notably liver, adipose tissue and brain. A re-setting of HPA axis sensitivity is thought to underlie many of the cardio-metabolic outcomes associated with low birth weight and glucocorticoid overexposure. Furthermore, many of the affective disorders that are linked to prenatal stress involve glucocorticoid-sensitive central nervous system targets. Excess glucocorticoid exposure in late pregnancy can also induce long-lasting effects on peripheral tissue expression of glucocorticoid-sensitive genes. In rat models of prenatal glucocorticoid exposure or maternal malnutrition, the hepatic glucocorticoid receptor is upregulated, plausibly driving the observed increase in both the expression and activity of the gluconeogenic enzyme phosphoenolypyruvate carboyxkinase (PEPCK), and programming insulin resistance and impaired glucose tolerance in adult life (Hales et al., 1996; Ozanne et al., 1996; Nyirenda et al., 1998).

It is clear that the effects of prenatal stress and glucocorticoid excess during pregnancy on subsequent physiological and psychological outcomes differ not only due to the timing of exposure, but also on the sex of the offspring. In human pregnancy, the placenta of female foetuses may impart a relative protection from glucocorticoid excess due to increased glucocorticoid inactivation compared with males (Clifton and Murphy, 2004). Recent data in the rat has also shown that male, but not female, offspring exposed to stress in early gestation exhibited increased anxiety-related behaviours as adults, again changes associated with sex-differences in placental function (Mueller and Bale, 2008). However, the precise mechanisms of sex-specific susceptibility to prenatal insults are not yet clear. Likewise, the issue of timing is clearly species-specific, and presumably related to the expression of GR and mineralocorticoid receptors (MR) in relation to the exposure.
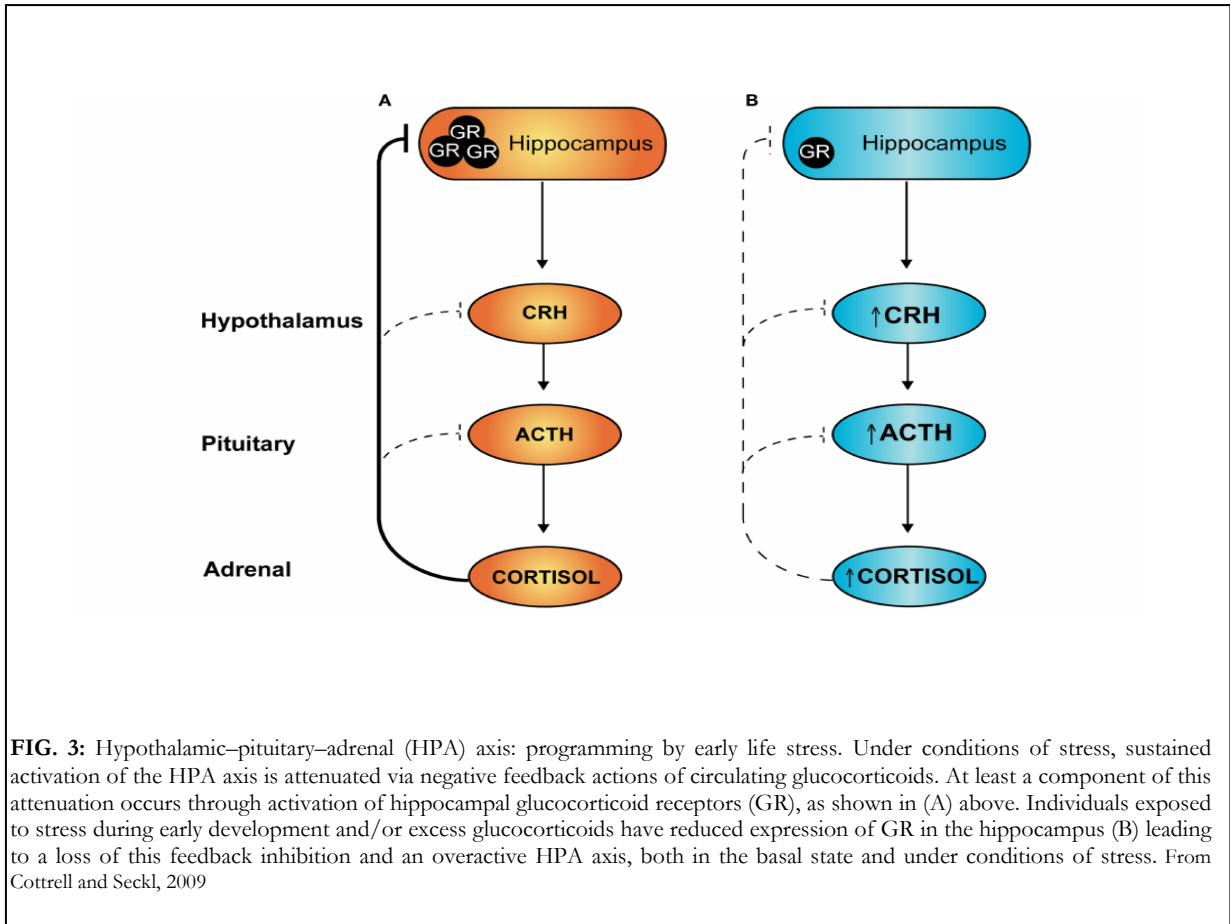
FIG. 3: Hypothalamic–pituitary–adrenal (HPA) axis: programming by early life stress. Under conditions of stress, sustained activation of the HPA axis is attenuated via negative feedback actions of circulating glucocorticoids. At least a component of this attenuation occurs through activation of hippocampal glucocorticoid receptors (GR), as shown in (A) above. Individuals exposed to stress during early development and/or excess glucocorticoids have reduced expression of GR in the hippocampus (B) leading to a loss of this feedback inhibition and an overactive HPA axis, both in the basal state and under conditions of stress. From Cottrell and Seckl, 2009

From the above discussion, it would appear that the programming of HPA function involves modification of glucocorticoid negative feedback at the level of the limbic system, hypothalamus and pituitary. Together these data indicate that the ascending serotonergic system is probably involved in glucocorticoid-induced HPA programming during prenatal life. However, this is clearly not the only route by which prenatal glucocorticoid exposure influences HPA function. Many studies have identified influences of prenatal stress and synthetic glucocorticoid on other central neurotransmitter systems that are involved in the regulation of HPA function (Weinstock, 2005).

Both prenatal stress and synthetic glucocorticoids can also influence the development and subsequent function of other neuroendocrine systems including the hypothalamo-pituitary-gonadal and the hypothalamo-pituitary-thyroid systems. Such modification will indirectly influence HPA function.

Further, both prenatal stress and synthetic glucocorticoids have been shown to have a long-term impact on brain structures, particularly in the hippocampus, which may in turn influence HPA function (for review see Owen et al. 2005). Thus, prenatal glucocorticoid exposure permanently increases basal plasma corticosterone levels in adult rats (Levitt et al., 1996; Welberg et al., 2000). This is apparently because the density of both types of corticosteroid receptor, GRs and MRs, are lastingly reduced in the hippocampus, changes anticipated to attenuate HPA axis feedback sensitivity. Maternal undernutrition in rats (Langley-evans et al., 1996) and sheep (Hawkins et al., 2000), also, affects adult HPA axis function, suggesting that HPA programming may be a common outcome of prenatal environmental challenge, perhaps acting in part via alterations in placental 11 β-hydroxysteroid dehydrogenase-2 (11β-HSD-2) activity, which is selectively down regulated by maternal dietary constraint (Langley-Evans et al., 1996; Bertram et al., 2001).

Consequent plasma glucocorticoid excess exacerbates hypertension and hyperglycemia in such prenatal environmental programming models (Langley-Evans, 1997). Tissue glucocorticoid action is further increased by the documented elevations in hepatic and visceral adipose tissue glucocorticoid sensitivity (Nyirenda et al., 1998; Cleasby et al., 2003).

Alterations in the regulation of HPA activity throughout life will impact on adult health due to altered tissue exposure to endogenous glucocorticoids. Chronically elevated plasma cortisol/corticosterone has been associated with atherosclerosis, immunosuppression, depression and cognitive impairment, as well as elevated

cholesterol levels and increased incidence of diabetes (Sapolsky et al. 2000; Lupien and Lepage, 2001).

At the level of the CNS, it is possible that a compromise in the developing hippocampus (i.e. reduction of pyramidal neurons or alterations in axonal/dendritic processes and synaptogenesis), associated with glucocorticoid exposure, may decrease the age at which hippocampal deficits (associated with normal ageing) are first noted. Prenatally programmed increases in HPA function will exacerbate this hippocampal deficit, and in turn lead to further increases in HPA function (due to reduced glucocorticoid negative feedback). In this regard, there is a reduction in the efficiency of glucocorticoid feedback as humans age, resulting in extended HPA responses to stress and elevated exposure to cortisol. This has been linked to a reduction in hippocampal volume and impaired cognitive ability (Lupien and Lepage, 2001). For these reasons, follow-up studies in humans and animal models, to investigate the impact of prenatal stress and synthetic glucocorticoid exposure, may fail to identify significant functional deficits in the hippocampus until adulthood or early old age.

Thus, the perinatal environment has persistent influences on developmental programming of interindividual differences in metabolic and endocrine function that contribute to emotional and cognitive performance through life. The alterations in the regulation of HPA activity, liable of long-lasting modification, are mediated by three critical components that are a crucial node between the maternal environment and the foetus: 11β-HSD2, glucocorticoid receptors and corticotrophin releasing hormone (Cottrell and Seckl, 2009).

- In normal pregnancy, maternal glucocorticoid levels are markedly higher than those in the foetal circulation. The foetus is protected from the relatively high maternal glucocorticoid levels of pregnancy by the placental inactivation of active glucocorticoids (cortisol in humans, corticosterone in rodents) to its inactive 11-

keto forms (cortisone and 11-dehydrocorticosterone, respectively) by **11β-HSD2**. This enzyme acts as a "barrier" to prevent premature or inappropriate action at glucocorticoid-responsive tissues during foetal development. It has been suggested that a reduction in the expression or activity of placental 11β-HSD2, by leading to increased transplacental passage of active glucocorticoids, reduces fetal growth. In support of this notion, in human and rodent pregnancy foetal weight is correlated with 11β-HSD2 activity (Stewart et al., 1995; Lindsay et al., 1996; Murphy et al., 2002). In addition, mutations in the HSD11B2 gene in humans, although rare, markedly reduce birth weight (Dave-Sharma et al., 1998).

Maternal circulation

Fetal circulation

Placenta

11β-HSD2

▲ **Active GCs**

● **Inactive GCs**
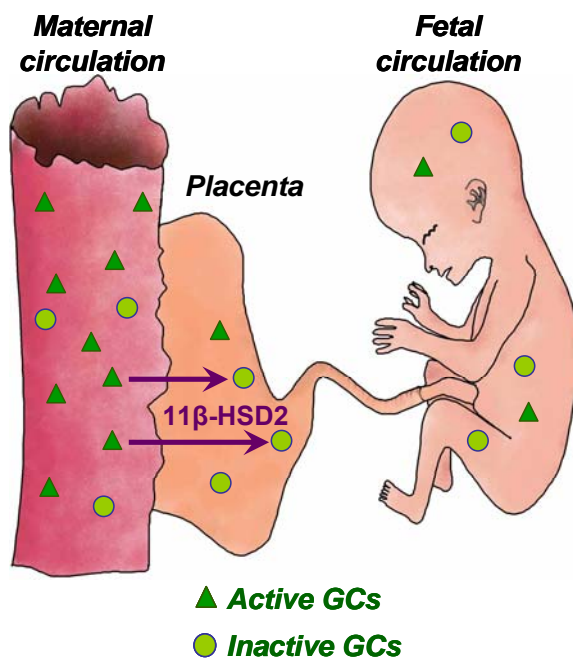
FIG. 4: The placental type 2 11β-hydroxysteroid-deshydroxygenases (11β-HSD 2), forms a foeto-placental barrier against maternal glucocorticoids. 11-HSD type 2 in the placenta and in many fetal tissues until mid-gestation rapidly inactivates cortisol (corticosterone in rats and mice) to inert cortisone (11-dehydrocorticosterone). This ensures the fetal environment has low levels of active glucocorticoids derived largely from the fetal adrenal glands. Individuals homozygous for deleterious mutations of the 11βHSD2 gene have very low birth weight. Natural variations in placental 11β-HSD2 activity correlate with birth weight in some studies in humans and rodents. Inhibition of 11β-HSD in pregnant rats reduces birth weight and produces permanent cardiovascular, metabolic, neuroendocrine and behavioural sequelae in the adult offspring. Adapted from: Seckl, 2001

Numerous studies have, also, shown that inhibition of 11β-HSD2 during pregnancy leads to the development of later hypertension and glucose intolerance (Edwards et al., 1993; Lindsay et al., 1996; Langley-Evans, 1997b), as well as programming increased HPA axis activity and anxiety-related behaviours (Welberg et al., 2000). More recently, genetic manipulations have shown that 11β-HSD2 knockout mice (11β-HSD2−/−) exhibit reduced birth weight and heightened anxiety in adulthood (Holmes et al., 2006).

Importantly, placental 11β-HSD2 may be a crucial node between the maternal environment and the foetus. Thus, maternal undernutrition or ingestion of a low protein diet (which also lower birth weight and leads to cardio-metabolic syndrome in the offspring) decreases the expression and/or activity of placental 11β-HSD2 (Langley-Evans et al., 1996 b; Lesage et al., 2001; Stocker et al., 2004). Maternal stress similarly reduces placental 11 β -HSD2 (Mairesse et al., 2007). In vitro studies using placental cells lines have also indicated that a number of other factors, including hypoxia, catecholamines and proinflammatory cytokines, can down-regulate 11β-HSD2 activity (Hardy and Yang, 2002; Chisaka et al., 2005; Homan et al., 2006). These findings are of particular relevance to human situations of placental insufficiency or maternal infection.

- During development, **GR receptors** (GRs) are expressed from early embryonic life in most tissues. GRs are essential for offspring survival, as indicated by the lethal postnatal phenotype of GRs null mice (Cole et al., 1995). The use of transgenic mouse models has identified that variation in the level of GRs alters stress responses and activity of the HPA axis. A 30–50% reduction in GRs is associated with exaggerated HPA axis responses to stress (Pepin et al., 1992; Michailidou et al., 2008), whereas the generation of transgenic GRs-overexpressing animals produces mice with a relatively stress resistant phenotype (Reichardt et al., 2000; Wei et al., 2004).

Exposure of the foetus to stress or high levels of glucocorticoids –whether from exogenous or endogenous origins – can permanently affect GRs expression. For instance, inhibition or deficiency of placental 11β-HSD2 has been shown to reduce hippocampal GRs expression (Levitt et al., 1996), but conversely increases amygdala GRs mRNA levels (Welberg et al., 2000). A reduction in hippocampal GRs would be expected to reduce glucocorticoid negative feedback and lead to an overactive HPA axis.

In rats, exposure to high levels of postnatal care (high levels of licking and grooming by the dam) leads to increased GRs mRNA expression in the hippocampus and reduced HPA axis responses to restraint stress as adults compared with offspring raised by low licking-grooming (low-LG) dams (Liu et al., 1997). Postnatal handling, which removes the differences in hippocampal GRs expression, eliminates these maternal programming effects (Meaney et al., 1989). In addition, cross-fostering studies indicate that the effect on GRs expression is directly related to the level of maternal care (Francis et al., 1999).

- A critical component of the central HPA axis, **corticotrophin releasing hormone** (CRH) has also been proposed as a mediator of the effects of early life stress on later cognitive and behavioural outcomes. In humans CRH is produced by the placenta and released into the maternal and fetal circulation (Goland et al., 1993). High concentrations of CRH are found in growth retarded foetuses, and elevated maternal CRH levels are associated with decreased gestational length and an increased risk of preterm delivery (Wadhwa et al., 1998).

In contrast to the negative feedback actions of glucocorticoids on the expression and release of central CRH, placental production is enhanced by glucocorticoids (King et al., 2001). Hence, maternal stress, accompanied by elevated CRH and glucocorticoid concentrations, affect both the length of pregnancy and the hormonal environment of the developing foetus. Administration of CRH to pregnant rat dams reduces both maternal body weight during pregnancy and offspring weight during the first two postnatal weeks, as well as enhancing offspring behavioural responses to a stressor in the early neonatal period (Williams et al., 1995), effects reminiscent of the actions of maternal stress (Williams et al., 1998). Therefore, it was suggested that at least a component of stress/glucocorticoid programming effects might be mediated through CRH actions on the developing fetal brain.

It is perhaps noteworthy that in terms of hippocampal development, the last trimester of human pregnancy is equated with the first postnatal week in the rat (Avishai-Eliner et al., 2002). Although detailed analysis of the ontogeny of GR expression in the human foetal brain has not been reported, in the rat there is a relatively high expression of GR from midgestation onwards (Diaz et al., 1998) and a rapid rise in GR and MR expression after birth (Matthews et al., 2002). Hence, the last week of gestation and early postnatal life are considered particularly sensitive periods in rodent development in terms of glucocorticoid-mediated programming. In support of this, handling of neonatal rat pups (an intervention which stimulates maternal care) reduced hypothalamic CRH expression and enhanced hippocampal GR expression in adult animals, and reduced the stress hormone responses to an acute stressor (Plotsky and Meaney, 1993; Avishai-Eliner et al., 2001).

1.3 Programming behaviour

Overexposure to glucocorticoids in utero leads to alterations in adult behaviours. Late gestational dexamethasone in rats apparently impairs coping in aversive situations later in life (Koenig et al., 2002). Prenatal glucocorticoid exposure, also, affects the developing dopaminergic system (Tronche et al., 1999; Levine 1957) with implications for understanding of the developmental contributions to schizoaffective, attention-deficit hyperactivity, and extrapyramidal disorders. Stressful events in the second trimester of human pregnancy are associated with an increased incidence of offspring schizophrenia (Meaney et al., 1988). Prenatal exposure to dexamethasone may exert more widespread effects because it also increases the susceptibility of the cochlea to acoustic noise trauma in adulthood (Meaney et al., 1989). Behavioural changes in adults exposed prenatally to glucocorticoids may be associated with altered functioning of the amygdala, a structure key to the expression of fear and anxiety. Intra-amygdala administration of

CRH is anxiogenic (Meaney et al., 1992). Prenatal glucocorticoid exposure increases adult CRH levels specifically in the central nucleus of the amygdala (Koenig et al., 2002; Liu et al., 1997). Prenatal stress similarly programs increased anxiety-related behaviours with elevated CRH in the amygdala (Smythe et al., 1994). Moreover, corticosteroids facilitate CRH mRNA expression in this nucleus (Mitchell et al., 1990) and increase GRs and/or MRs in the amygdala (Koenig et al., 2002; Liu et al., 1997). The amygdala stimulates the HPA axis via a CRH signal (O'Donnell et al., 1994). So, an elevated corticosteroid signal in the amygdala due to hypercorticosteronemia in the adult offspring of dexamethasone-treated dams may produce increased CRH levels in adulthood. A direct relation between brain corticosteroid receptor levels and anxiety-like behaviour is supported by the phenotype of transgenic mice with selective loss of GR gene expression in the brain, which shows markedly reduced anxiety (Yau et al., 1994).

2. PRENATAL RESTRAINT STRESS: AN ANIMAL MODEL TO STUDY DEPRESSION SYMPTOMS

2.1 PRS and HPA axis

Every disturbance in the body, either real or imagined, evokes a stress response, which serves to restore homeostasis and to facilitate adaptation. In concert with other components of the stress response system, the action of GCs displays two modes of operation. In the first, permissive (or "proactive") mode, GCs maintain basal activity of the HPA axis, control the sensitivity or threshold of the system's response to stress, promote coordination of circadian events, such as the sleep/wake cycle and food intake and are involved in processes underlying selective attention, integration of sensory information and response selection. In the second, suppressive (or "reactive") mode, GCs feedback helps to terminate stress-induced HPA activation. The steroids facilitate an animal's ability to cope with, adapt to and recover from stress.

A brief period of controllable stress may be experienced with excitement and can be beneficial to emotion and health. In contrast, lack of control and the uncertainty can produce a chronic state of distress, which is believed to enhance vulnerability to disease (de Kloet et al. 1993; 1998). In order to maintain stress responsiveness, both rapid activation and rapid inhibition of the stress response are necessary. Failure to activate the stress response places the organism in a very fragile state. But, an inability to inhibit the stress response, once initiated, increases the vulnerability to diseases, and, particularly, during development, may result in permanent effects on growth and differentiation of a number of systems, including the central nervous system (de Kloet et al., 1988).

Exposure to PRS has been shown to result in increased responsiveness of the HPA axis to stress (Henry et al., 1994, Maccari et al., 1995; Vallée et al., 1997; Morley-Fletcher et al., 2003a, b). PRS increases stress-induced corticosterone

secretion in preweaning rats (Henry, 1994) and induces a prolonged post-stress corticosterone secretion in adult animals (Fride et al. 1986). Increase of the corticosterone plasmatic levels, after activation of HPA axis, can be liable for the neurogenesis reduction since it has been shown that the corticosterone prevents the cellular proliferation (Cameron and Gould, 1994; Gould et al., 1992). Several studies demonstrate that preventing the corticosterone increase (through the surrenalectomy), stimulated by the stressful event (predator odor), it is possible to block the neurogenesis reduction (Tanapat et al., 2001) and in this way the hippocampal atrophy, induced by stress, is prevented. These data are very interesting because, recently, several review articles have presented the exciting new theory that clinical depression in humans could be related to a disturbance in neuronal plasticity and in hippocampal neurogenesis in particular.

The hyperactivity of the HPA axis is among one of the more relevant hormonal features observed, at least in a subpopulation of depressed subjects (Holsboer et al., 1984). It is characterised by hypersecretion of cortisol (Sachar et al., 1973; Rubin et al., 1987), resistance to dexamethasone test (Arana and Mossman, 1988) and increased levels of Corticotropin-Releasing Factor (CRF) in the cerebrospinal fluid (Nemeroff et al., 1984), and by decreased level of GRs (Webster et al., 2002).

PRS reduces, in addition, the metabotropic receptors in the hippocampus (Zuena et al., 2008), they are involved in the control of the HPA axis activity (Holsboer and Barden 1996; Scaccianoce et al., 2003) and in the mechanisms of synaptic and neuronal plasticity through activation of intracellular pathway resulting in the CREB phosphorylation Compiling pharmacological evidence implicates metabotropic glutamate (mGlu) receptors in the regulation of mood and sleep disorders suggesting that they may serve as novel targets for the discovery of small molecule modulators with unique antidepressant properties (Spooren and Gasparini, 2004; Ahnaou et al., 2009). For example, mGlu receptor modulation can

facilitate neurogenesis and the release of neurotransmitters that are associated with treatment response to depression in humans.

Levels of both types of corticosteroid receptors (mineralocorticoid, MR and glucocorticoid, GR) are reduced in the hippocampus of adult offspring, revealing a possible mechanism for the deficit of HPA axis feedback processes (Maccari et al., 1995, Van Waes et al., 2006).

Moreover, PRS accelerates age-related alterations in the HPA axis. Indeed, the period of hyporesponsiveness of the HPA axis is abolished in newborn PRS rats (Henry et al., 1994) and circulating glucocorticoid levels are increased in middle-aged PRS animals when compared to those of controls (Vallée et al., 1999), in fact, their levels are similar to those found in old animals.

Female PRS rats, after exposure to an intense inescapable footshock, durably exhibit attenuated corticosterone secretion after stress (Louvart et al., 2006). In males, PRS also results in the hyporesponsiveness of the HPA axis when animals are exposed to an alcohol challenge (Van Waes et al., 2006). These results suggest that the HPA alterations induced by PRS may vary in according to gender, as well as the nature (processive versus systemic/generalized) or the intensity of the stressor. The hyperactivity of the HPA axis observed in both male and female PRS rats is accompanied in adult rats by increased anxiety-like behaviour in males (Poltyrev et al., 1996; Vallée et al., 1997; Viltart et al., 2006), and an increased behavioural response to novelty in both males and females (Thompson, 1957; Fride et al., 1986; Wakshlak and Weinstock, 1990, Vallée et al., 1997; Louvart et al., 2006).

Although these data provide evidence for an impairment of negative feedback control of HPA axis in PRS rats, they suggest, also, that the change in corticosterone receptors is the result of the elevation of circulating GCs and not its cause. This could occur through a failure of the normal adaptation process, 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).

The cerebral reactivity to stress of brain areas implied in the feedback of the HPA axis in adult PRS rats remains unknown.
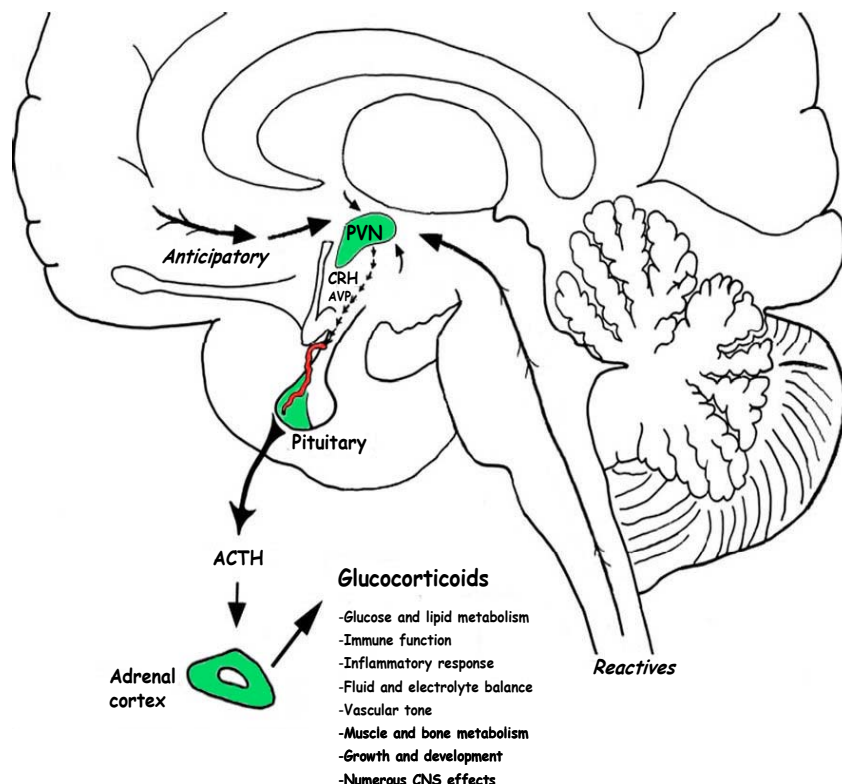
FIG. 5: Overview of the hypothalamo–pituitary–adrenocortical axis, including principal classes of regulatory afferents and corticosteroid actions. CRH (corticotropin releasing hormone) and AVP (arginin vasopressin) neurons located within the medial hypothalamic paraventricular nucleus (PVN) drive pituitary 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 Herman et al., 2003).

2.2 Neurochemical alteration induced by PRS

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, given that there is a reciprocal influence between these two systems (Joels et al., 1991; de

Kloet et al., 1998). PRS rats show an increased expression of the 5-HT$_{1A}$ receptors in the cortex (Morley-Fletcher et al., 2004) and in the hippocampus (Hayashi et al., 1998). In addition, PRS rats present an increase of the post-synaptic 5HT-2 receptors (Peters, 1986; 1988; 1990). Another very recent study shows a decreased levels of 5-HT$_{1A}$ immunobinding in the ventral hippocampus, which is primarily implicated in emotional processing, and this decrease is 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 CRF. The last hormone has been found to be increased in the amygdala (Cratty et al., 1995).

Reduced contents and lower turnover of noradrenalin (NAd) 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 the PRS eliminates the asymmetries in striatal dopaminergic function and reduces the asymmetries in the size of cerebral cortex (Alonso et al., 1991; 1994; 1997; Fleming et al., 1986). Like human subjects, normal rats display cerebral asymmetries which are related to patterns of brain organisation and control of various behavioural functions (Carlson and Glick, 1989). These specific alterations induced by gestational stress have been proposed to draw parallel with the clinical observations of the interference with the reduction of cerebral asymmetries observed schizophrenic patients (for this issue, see 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).

The noradrenergic neurons of the locus coeruleus constitute the central component of the sympathetic nervous system, that is involved in the stress response as in the attentional processes (for review: Morilak et al., 2005). At the central level, PRS increases the hypothalamic basal NA concentrations (Peters, 1982), while, at the plasmatic level, PRS rats shows the same basal NA levels than control rats (Weinstock et al., 1998) but, after an electric shock, they present an increased concentration of NA and of its metabolites (Weinstock et al., 1998b). This suggests a hyperactivity of the NA system in PRS rats.

The basal and stress response activities of the locus coeruleus noradrenergic neurons remain to be determined.

Few works have explored the glutamatergic and GABAergic systems in PRS rats. A recent study shows that PRS rats present an increase of NMDA receptors expression in the cortex, hippocampus and in various striatal areas (Shepherd et al., 2002).

2.3 Behavioural alterations

Exposure to PRS in the human beings and in the animals induces long-lasting effects on the behaviour, causing a general impairment of the adaptive capabilities of the individual (for an extensive review, see Weinstock, 2001).
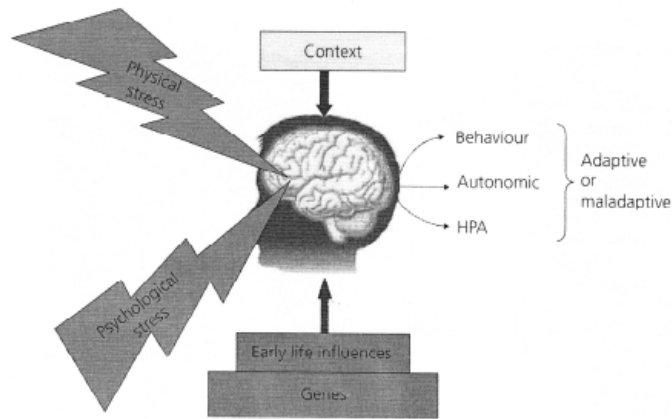
FIG. 6: Physiological and pathological responses to stress. The resilience or vulnerability of any one individual to stressul situations in adulthood will depend upon that genetic influence and early life experiences. Adapted from: Lightman, 2008

In animal studies, stress during pregnancy disrupts the normal course of sexual differentiation, reducing 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 behaviour to levels expressed by females (Ward and Stehm, 1991), thus eliminates the sex differences that are normally observed for this behavioural pattern (Meaney, 1989). PRS monkeys display much less play behaviour and exploration of their surroundings than control animals and this has been shown to be accompanied by more 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 children, prenatally stressed, 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. Adult PRS rats (Ward and Weisz, 1984; Wakshlask and Weinstock, 1990; Poltyrev et al., 1996; Vallee 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, Vallee and co-workers (1997) have demonstrated that the effects of PRS on several features of emotional behaviour are strongly correlated with post-stress levels of corticosterone. Thus, the alterations of the HPA axis activity induced by PRS may be associated to the changes observed in adult behavioural reactivity.

PRS, also, produces important modifications piggyback anhedonic-like behaviours and anxiety-like behaviours. The consumption of appetitive substances, like sweetened water, is used to model and to evaluate the anhedonia-like symptoms, characteristics of the depression (Katz 1982). Indeed, reduced sucrose consumption is 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). The PRS decreases the sucrose consumption in the females and increases it in males (Keshet et al., 1995). The behavioural response in the Porsolt test (1978) was validated as good index of the depressive-like behaviour. This test, also called "the forced swimming test", uses the concept of passive coping strategy in a uncontrollable situation, strategy which can be described as learned resignation. Several studies highlight that the PRS exacerbates the phenomenon of learned resignation in response to uncontrollable electric shocks (Secoli et al., 1998) and increases time spent in immobility in the Porsolt test (Alonso et al., 1991a; Drago et al., 1999; Morley-Fletcher et al., 2003; 2004), indicating that PRS rats have a higher propensity to behavioural inhibition in the situations of intense stress. PRS rats show, also, an increased fear and anxiety that appear by a reduction of exploration and an increase of the defecation in the open field test (Archer et al., 1971; Vallee et al., 1997; Weinstock et al., 1992). However, other studies show a behavioural hyperactivity, in particular, during the first minutes of various behavioural tests as the Y maze (Deminiere et al., 1992). *The differences of experimental conditions and the variety behavioural tests used could contribute to these various*

results. In the elevated plus maze, PRS rats spend less time and make less entry in the open arms, indicating an increased anxiety-like behaviours (Poltyrev et al., 1996; Vallee et al., 1997; Wakshlak and Weinstock, 1990; Rimondini et al., 2003; Zimmerberg et al., 1998). In the test of the black and white box, the PRS induces a reduction in the number of entry and spent time in the white compartment (Ward et al., 2000). The increased anxiety-like behaviour is, also, detectable by a reduced social interaction in PRS young rats (Ward et al., 1991; Morley-Fletcher et al., 2003b) and in adult females rats (Weinstock, 2001).

Moreover, alterations of mnemonic and attentional performances are induced by PRS. Several studies are interested in the mnemonic performances of PRS rats. The prenatal stress affects the spatial learning in the adult male rats in the swimming test (Morris test), a hippocampal-dependent test (Lemaire et al., 2000) and in the water-filled T-maze (Nishio et al., 2001). Moreover, PRS influences 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, the PRS provokes the contextual fear conditioning increasing the conditioned response of immobility (Shalev et al., 2001). However, it delays the learning of passive (Drago et al, 1999) and active avoidance tasks (Lehmann et al., 2000). These alterations could be induced by attentional deficits. Latent inhibition paradigm can be used for assess the attentional performance (Solomon et al., 1980). Although several studies show that latent inhibition are not affected by PRS (Shalev et al., 2001), other works suggest a sex-specific effect with an increased latent inhibition in males PRS rat (Bethus et al., 2005). According to Lemaire and co-workers, the mnemonic effects induced by the PRS would be determined by a decreased hippocampal neurogenesis during the learning (Lemaire et al., 2000), but this work were realized only in the male rats. *The comparison of the effects of the prenatal stress between the male and female rats on the neurogenesis and on the factors controlling it could make to better understand the mechanisms of*

these deteriorations and their functional consequences. In addition, several works have observed the mnemonic performances of old PRS rats and they have showed that the ageing is implied at the same time in a reduction of the hippocampal neurogenesis and in a decrease of the mnemonic performances. Thus, Valley and co-workers demonstrated that the PRS improves the mnemonic deficits related to the age during a test of spontaneous space recognition in male rats (the Y maze), but have no effect on the Morris water maze test performances (Vallee et al., 1999). On the other hand, Darnaudéry and co-workers proved that a PRS increases the mnemonic deficits related to ageing in 24 months old female in the Morris water maze test (Darnaudéry et al., 2006). The effects of a PRS on the mnemonic performances stay incompletely characterized.

2.4 Circadian rhythms

Many of the physiological or behavioural 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, 1996). 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, circadian functions and sleep patterns are also regulated by neurochemical or behavioural stimuli (Van Reeth and Turek, 1989). Among these stimuli, steroids have a marked effect on the functioning of the circadian system (Turek and Gwinner, 1986) and chronic stress in adult rats can induce changes in circadian rhythms as well as in sleep patterns (Kant et al., 1995; Cespuglio et al., 1995).

In this regard, it has been shown that, in rats, PRS 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 mineralocorticoids (MRs) levels at the beginning of the light phase have been observed for males and at the end of it for both sexes (Koehl et al., 1999). As a whole, PRS rats exhibit an altered temporal function of the HPA axis reinforcing the idea of a general homeostatic dysfunction in those animals.

Disturbances in the circadian rhythm of locomotor activity have, also, been reported. 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).

An 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. 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 could be a good alternative, because exposure to high glucocorticoid levels or acute stressors results in significant alterations in 5-HT turnover in the midbrain/pons area in PRS rats (Muneoka et al., 1997). As already reported, PRS in rats induces long-term altered response to 5-HT receptor agonists (Peters, 1988). In view of the permissive 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.

This altered pattern of circadian rhythms together with an overall impairment of the HPA axis activity observed in PRS rats, parallels with the abnormalities in circadian rhythm that have been documented in a great majority of depressed patients (Rosenwasser and Wirz-Justice, 1997), suggesting that the PRS rat may be an animal model to study these symptoms of depression.

2.5 Assessment of the PRS as a model of depression

It is possible to affirm that a model is a simplified representation of a system very more articulated. If to the word model is added animal, the things are complex because the purpose of the "construction" of an animal model is that to have an experimental substratum that tries to simulate a human illness and to study of it (I) the aetiology, (II) the physiopathology, (III) the symptomathology and (IV) the response to the treatment (usually pharmacological). It is more elaborate when to the definition "model animal" it is added the words "in psychiatry" because the illness that must be reproduced in the animal is tightly a human illness and, therefore, it not present in other species.

The animal model of depression presents just the symptomathology and the response to the treatment, because it is not possible to know the aetiology and the physiopathology of this illness. Moreover, an animal model of depression can reproduce only some of the symptoms that characterize the human illness.

Willner (1997) proposed three criteria that animal models of depression must exhibit: *face*, *predictive* and *construct* validity. Face validity refers to the phenomenological similarity (how much the symptoms observed in the animal model resemble to those of the human patients), 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 criterion that in some models is considered satisfied, is difficult to reach really because it is founded on the knowledge of the causes of the psychiatric illnesses.

However, because mental disorder is a human pathology, 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 some similar symptoms and develop new pharmacological strategies.

- **Face validity**. Several studies have clearly 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 (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 human beings (Poland et al., 1992), PRS adult rats present persistent changes in sleep architecture that parallel those found in depressed patients (Dugovic et al., 1999). Moreover, 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). In this regard, it is important to understand that the persistence of paradoxical sleep alterations observed in the PRS model is 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.

Recently, it has been shown that PRS, in rat, to induce lifespan reduction of neurogenesis in the hippocampus (Lemaire et al., 2000). This is in accordance with stress-induced structural remodelling in the hippocampus that can characterise the impairment of neural plasticity in the brain of depressed patients (Sheline et al., 1996; Sapolski, 2000; McEwen, 2001).

From a behavioural point of view, PRS rats exhibit anxiety behaviour (Vallee et al., 1997; Weinstock et al., 2001) and comorbidity with anxiety is often characteristic of human depression (Stahl, 1993; Rouillon, 1999). Furthermore, Alonso and co-workers (1991; 1997) have shown that PRS female rats exhibit behavioural 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 (Vallee et al., 1999). In the light of these, it is possible to affirm 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 AG0310 (a dual antidepressant with melatonergic agonist and 5-HT$_{2C}$ antagonist properties) reverse several PRS-induced alterations at the behavioural, 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 densities in the hippocampus and, modified 5-HT_{1A} mRNA expression (Morley-Fletcher et al., 2003, 2004). 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 (Darnaudery et al., 2006), and a chronic AG0310 treatment increases hippocampal neurogenesis especially in the ventral part of the hippocampus of male rats (Maccari et al., 2005). These finding gives further support to the validity of the PRS model. 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.

It is possible conclude that the PRS rats respond positively to antidepressant treatment, this indicates a good predictive validity of the model, therefore it can be a good tool to test new pharmacological strategies to treat mood and sleep disorders.

- **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 at moments when critical developmental processes are taking place in parts of the nervous system or neuronal circuits involved in (later) HPA-axis functioning may induce in some individuals distinct and stable patterns of dysregulations that are associated with altered emotional processing and heightened responsiveness to stress.

Finally, all previous points support the validity of the PRS model as an excellent animal model of anxiety/depression.

3. PHARMACOLOGICAL TREATMENT OF DEPRESSION

3.1 Antidepressants and their action mechanisms

The persistence of the anomalies induced after the prenatal stress in our animal model can be interesting to test new pharmacological strategies concerning the mood and sleep disorders. Indeed, contrary to many other animal models, the symptoms presented by PRS rats are installed in time and, thus, allow the study of the effects of chronic pharmacological treatments classically used in the depressive disorders.

Several work completed in our laboratory, thus, attempted to determine the effects of chronic antidepressants treatment on PRS rats. In the Porsolt forced swimming test the PRS increases the immobility behaviour and decreases the swimming. The plasmatic corticosterone levels were positively correlated with the immobility and negatively with swimming (Maccari et al., 2001). PRS rats chronic treatment with a tricyclic antidepressant such as the imipramine (3 weeks daily, 10mg/kg i.p.) decreased the immobility in this test (Morley-Fletcher et al., 2003; 2004a). In order to extend this study, the effect of the treatment was examined on other parameters. This chronic antidepressant treatment attenuated the deteriorations of the social behaviour observed in PRS rats. The decreased GR and MR expression observed in the PRS rat hippocampus was alleviated by the antidepressants treatment (Morley-Fletcher et al., 2004b). In addiction, a new antidepressant, AG0310 is able to reverse the PRS alteration on hippocampus neurogenesis (Morley-Fletcher et al., unpublished data). Moreover, the majority of the effects chronic antidepressant treatment observed in PRS rats were not observable in control rats receiving the same treatment. These results indicate that

PRS animal are more sensitive to the effects of the antidepressant treatment than control rat. Previos data reinforce the idea that PRS rats constitute a good instrument to develop and test new pharmacological strategies to treat depression.

Thus, the antidepressant pharmacological treatment has as objective to alleviate the illness symptoms. The first antidepressants, introduced in the '50 and '60 years, are, respectively, the inhibitors of the monoamine-oxidase (IMAO) and the tricyclic antidepressants (TCA) (Alpers and Himwich, 1972). These drugs have been used for the depression treatment for many years, and their efficacy has been and it is broadly documented; nevertheless, the collateral effects that they produces have stimulated the research of new drugs better tolerated and less toxic.

Stress had been shown to alter normal serotonergic and dopaminergic neurotransmission (Piazza and LeMoal, 1996; Meijer and de Kloet, 1998) and, in animal models of depression, the efficacy of drugs that act on the serotonergic system in reversing some of the stress induced effects suggests that serotonin may be involved in their development or expression (Grippo et al., 2006; Muscat et al., 1992; Willner et al., 1987). In fact, after decades of slow progress, in the '80 years emerge new antidepressants classes: the selective serotonin reuptake inhibitors (SSRI) as fluoxetine, sertalin and fluvoxamin; the serotonin-noradrenalin reuptake inhibitors (SNRI) as the venlafaxin; noradrenergic and specific serotonergic antidepressant (NaSSA) as the mirtazapin; norepinephrine-dopamine reuptake inhibitor (NDRI) as the bupropion. Although the effectiveness of these new drugs does not overcome that of the precedents, their safety and tolerance have brought about their rapid diffusion.
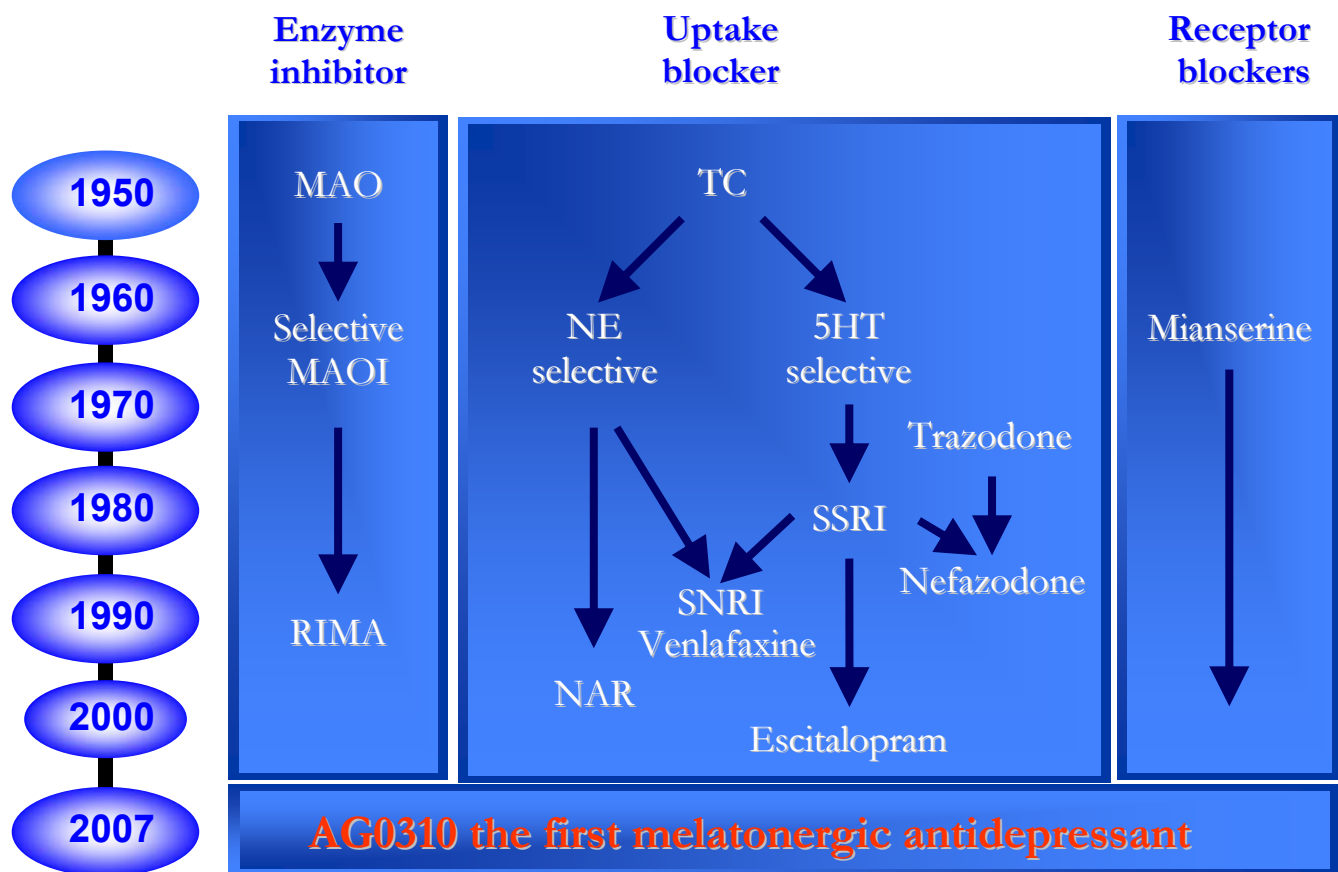
FIG 7: Schematic view of evolution of antidepressant drugs since '50 years at nowadays.

The drugs choice, for the depression treatment, depends on a lot of variables among which the most important is the patient response: the SSRIs, the venlafaxin, the mirtazapin and the bupropion are largely approved as first-option drugs and are currently the most commonly prescribed antidepressants in all age groups. This is due mainly to their relative safety and better side effect profile, as compared to tricyclic antidepressants and monoamine oxidase inhibitors. On the other hand, the TCS and from last the IMAO are usually reserved to those patients that do not respond to the therapy with new drugs (Expensive ICSI Health Guideline, 2004).

The knowledge of the pharmacological properties and the action mechanisms of antidepressants remain yet incomplete. The antidepressants, independently from the affiliation class,

induce an increase of the sinaptic availability of aminergic neurotransmitters, noradrenalina (NA), dopamin (DA) and/or 5-HT that stop the neuronal reuptake, reducing the catabolism through the MAO inhibition, removing the inhibitory tone on the release or on the neuronal activity.

The main effect of tricyclic is the non selective block of the monoamine reuptake from the nervous terminations, probably due to the competition for membrane transport: NAT, SERT and DAT (Blakely et al., 1994; Miller et al., 1999). The most important problem in the tricyclic use, as also in the IMAO, is the great numbers of remarkable collateral effects (faucis dryness, constipation, obfuscated vision, tremors, cardiotoxicity) consequent to their action on different neurotransmitter systems (mainly the colinergic, histaminergic and adrenergic). The SSRI are powerful selective inhibitors of the 5-HT neuronal reuptake and, to the doses normally used, they do not have important interactions with the other neurotransmitters; this greater 5-HT selectivity explains the reduction of the collateral effects in comparison to the tricyclics (Baldessarini, 1989; Stahl, 1998).
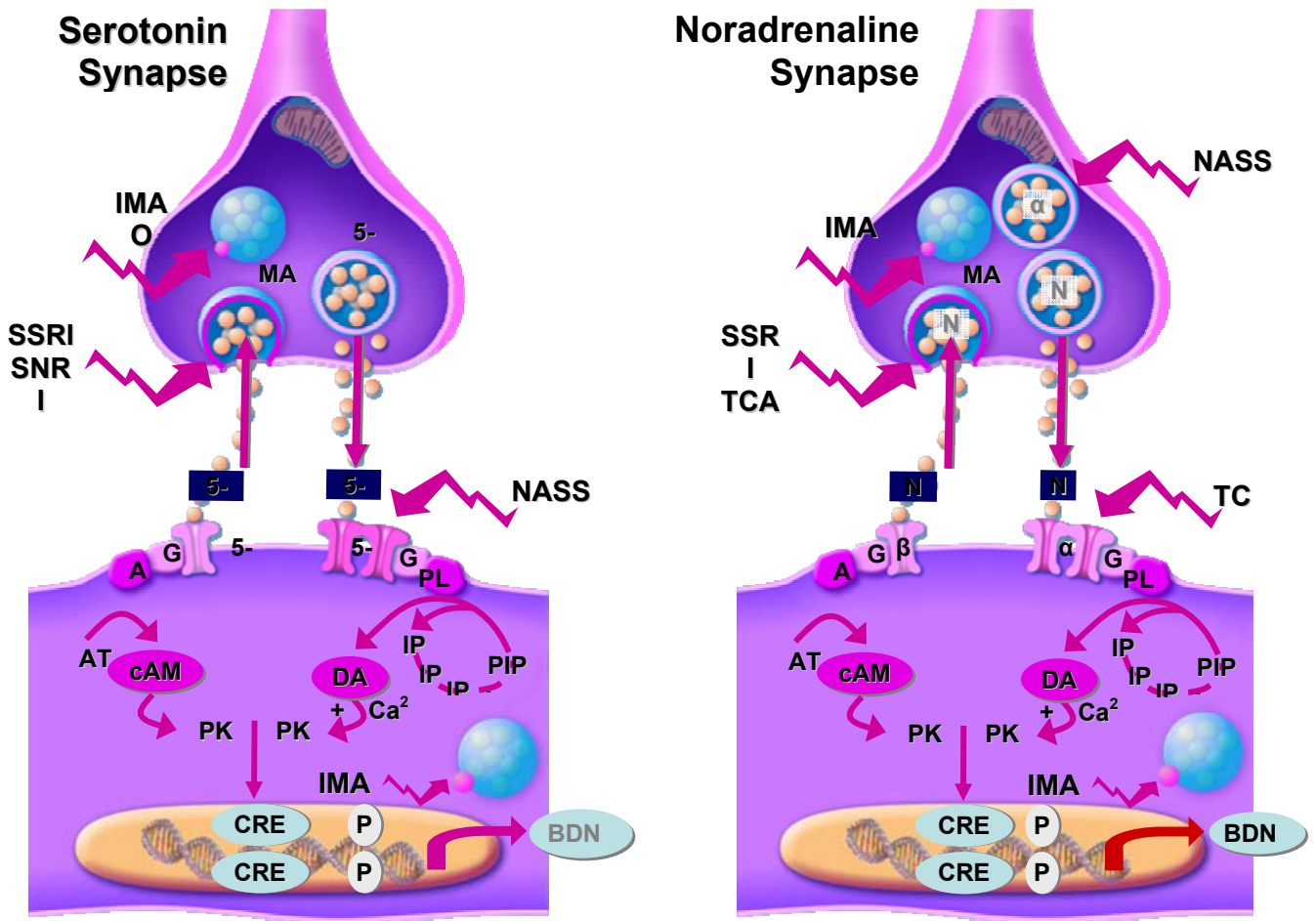
FIG. 8: A schematic view of sites of action of antidepressants.

The new antidepressants as the venlafaxine, the mirtazapine and the bupropion, said "atypical", have an action mechanism similar to that of the SSRI with different selectivity for the monoamines, and, show, also, the similar collateral effects.

The development of new more effective therapies is necessary for two fundamental questions linked to the treatment with the available antidepressants. The first problem is that the delay, in the onset of the drugs effects (Blier and de Montigny, 1994) could contribute to the great percentage (15%) of suicides among the depressed patients (Mann et al., 2005). The latter is that, among the depressed patients, exists a great variability in the sensibility to the antidepressant treatment, as

pointed out by the fact that at least one-third of the patients do not respond to antidepressant treatment (Joyce and Paykel, 1989; Quitkin et al., 1996).

The research of new more effective antidepressants has leaded to the experimentation of a new molecule, the AG0310. The AG0310 is a specific agonist of the MT1 and MT2 receptors of the melatonin (Ying et al., 1996) and also a selective antagonist of the 5-HT$_{2C}$ receptors of the serotonine (Millan et al., 2003). As agonist, the AG0310 mimes the effects of the melatonin on different systems (Ying et al., 1996; Martinet et al., 1996): for example chronic administrations of AG0310 in the rat are able to resynchronize an altered circadian rhythm (Van Reeth et al., 1997). Moreover, preclinic studies has recently underlined that the AG0310 is able to induce antidepressants effects in some animal models of depression. In the model of chronic moderate stress, in the rat, the chronic treatment with AG0310 produces the same effects on the sugar intake of the imipramina and the fluoxetine (Papp et al., 2003); besides, the AG0310 influences the immobility time comparable to those of the imipramine in the model of the forced swimming, in the rat, (Bourin et al., 2004) and, preliminary studies point out that it would seem to be also effective in the model of the learned helplessness (Bertaina-Anglade et al., 2002).

Recent clinical studies (Loo et al., 2002; Kennedy and Emsley, 2005) show that the AG0310 has effectiveness similar to that of the classical antidepressants, also, in patient affected from severe depression and, besides, it possesses a good tolerance.

It is possible observe the therapeutic effects of antidepressants only after different weeks of continued assumption (Blier and de Montigny, 1994). To explain this delay it is necessary to hypothesize that a phenomenon of receptorial "adaptation" is established: after a long treatment with antidepressants and to the consequent monoamine accumulation at synaptic level, a desensitization of catecolaminergic and serotoninergic receptors would be verified (Blier and Montigny, 1998).

3.2 Role of antidepressants on neuroplasticity

It has been possible to hypothesize a new action mechanism of antidepressant after that the neurogenesis has been discovered in the adult and after that the experimental verification have showed that the antidepressants stimulate the cellular proliferation (Santarelli et al., 2003; Malberg et al., 2000) Insofar, the delayed action of the antidepressant treatment could be explained with the necessary time to the birth and the differentiation of new neurons that corresponds, in fact, to the time for the onset of the clinical effectiveness of drugs (Malberg and Schechter, 2005). It is interesting, moreover, the fact that, in the rat, the chronic treatment, but not that acute, with antidepressants increases the brain-derived neurotrophic factor (BDNF) (both the mRNA and the protein) in different limbic structures (Nibuya et al., 1995, 1996; Okamoto et al., 2003). BDNF increase leads to an increase of the CREB phosphorylation (cAMP response element). pCREB (phosphoCREB) is one of principal effector of the intracellular pathway activated by the BDNF (for review: Malberg et al., 2005). Furthermore, BDNF increase after chronic treatment with antidepressants would coincide with the onset of the therapeutic effects, and this suggests that the action of the antidepressants could be mediate really from the BDNF. This hypothesis is confirmed by the observation that for the onset of the antidepressants effects it is necessary the activation of the TrkB receptors (receptors that link the BDNF) (Saarelainen et al., 2003). Therefore, the ability of new antidepressant to induce cellular proliferation and neurogenesis can represent an innovative strategy for the discovery of new molecules. In addition, it is possible hypothesize that the alterations in hippocampal neurogenesis may play a role not only in the treatment of depression, but also, in the pathology (Jacobs et al., 2000). This hypothesis is attractive for understanding age-dependent antidepressant responses that supports the idea that there is increasing evidence that the magnitude

of hippocampal neurogenesis decreases with age (Kuhn et al., 1996; Nacher et al., 2003; Rao et al., 2006).

4. SPECIFIC AIMS OF THE THESIS

Brain function and dysfunction throughout life are determined by the interaction of genetic factors with "acquired" environmental events, signals and stimuli (McEwen, 1999). Genetic factors contribute crucially to brain function, whereas events that occur early in life are capable of exerting effects that persist throughout adulthood, as altered **NEUROPLASTICITY**; among these events that exert long-lasting influence on brain function, exposure to stress during critical period of development has significant place.

Stress triggers molecular cascades that allow rapid behavioural, autonomic and cognitive CNS responses to stressuful circumstances, followed by prompt re-establishment of functional steady-state. This involves not only rapid secretion of effector molecules, but also a more protracted, coordinated change in programmed gene expression (Avishai-Eliner et al., 2002).

We have used, in this study, a model of **PRENATAL RESTRAINT STRESS,** a rat model of early stress that, as previously shown, results in long-term alterations of neuronal functions and in the development of long-term biochemical, endocrinological and behavioural disorders. The impact of PRS is already detectable at foetal stage, giving further support to prenatal stress programming in adult pathophysiology. The studies so far conducted by our group and others indicate that the face as well as the predictive value of the PRS model is high, since several abnormalities observed in the PRS rat parallel those found in human depression (Maccari et al., 2003; Maccari and Morley-Fletcher, 2007). These are evidently concerned with the neurobiological symptoms of depression, and not the syndrome of depression, which includes mood aspects that clearly cannot be reproduced in the rat model. In fact, it is quite obvious that the aetiology of a complex psychiatric disease like depression in humans cannot be reduced merely to a few biological changes exhibited by rats. Despite this consideration, the utility of an animal model

lies in its capacity to augment our knowledge of the mechanisms underlying the actions of antidepressants on specific symptoms, with the goal of developing more appropriate and better targeted drugs and to increase their efficacy on those specific symptoms. Interestingly, in the PRS rat animal model, specific abnormalities can be effectively reversed by chronic antidepressant treatment.

Objectives

All the neurobiological studies have been conducted on the **HIPPOCAMPUS** because it is a key region in the regulation of stress response and neuroplasticity and it has become a promising target for the action of antidepressants.

a) The first aim of this PhD thesis was to better characterize the **PHENOTYPE** of PRS rats investigating both behavioural and neurochemical levels, to increase its face value and to discover new possible parallel with depression, fundamental elements to approach new pharmacological therapies.

To reach our goal we tried to answer to following questions:

1) does the PRS rebound on **circadian rhythms**, in particular on locomotor activity, on sleep/wake cycle and on daily CRH expression?

Lately, several studies have confirmed the new hypothesis that alterations in sleep and intense locomotor activity contribute importantly to processes of decrease of hippocampal neurogenesis that could provoke the onset of depression.

2) does the PRS have consequences on hippocampal **neurogenesis** and **candidate proteins**?

It is seemed interesting to study the neurogenesis for two relevant reasons: first of all, it is well-known that one of the most potent inhibitors of neurogenesis is stress; stressors that are either prolonged or extreme, may result in abnormal changes in brain plasticity; second, in recent years, several works sustain the hypothesis according to which an alteration of brain plasticity, particularly of hippocampal

neurogenesis would be the cause of neurobiological degeneration typical of depression.

3) does the PRS have effects on the ontogenesis of **metabotropic glutamate receptors**?

We have decided to study these receptors, involved in neuronal and synaptic plasticity, because accumulating evidence from biochemical and behavioural studies support the idea that the regulation of glutamatergic neurotransmission via mGlu receptors is linked to mood and sleep disorders and that these receptors may serve as novel targets for the discovery of small molecule modulators with unique antidepressant properties. For example, mGlu receptor modulation can facilitate neurogenesis and the release of neurotransmitters that are associated with treatment response to depression in humans.

b) The second aim of this PhD thesis was to confirm the predictive value of PRS model and to test antidepressants that are more appropriate and effective to reverse specific symptoms of depression. We analyzed the capability of a new antidepressant, **AG0310**, and **fluoxetine (Prozac®)** to reverse the PRS alterations respectively on circadian rhythms and on brain plasticity. .

In this second section we aimed to answer to following questions:

1) is the AG0310 able to reverse alterations provoked by PRS on circadian rhythms in particular on sleep/wake cycle and on locomotor activity?

We aimed to establish if the AG0310 can normalize altered circadian rhythms induced by prenatal stress, improving REM sleep and locomotor activity, because depressed patients commonly experience circadian rhythm desynchronization as sleep dysfunction associated with HPA axis hyperactivity. AG0310 is an agonist of the melatonergic $MT_{(1)}$ and $MT_{(2)}$ receptors, as well as a $5\text{-}HT_{(2C)}$ receptor antagonist

and it can be an innovative pharmacological approach in depression, considering, also, that it has a good efficacy and it is generally well tolerated.

2) is the fluoxetine able to correct the abnormalities caused by PRS on hippocampal neurogenesis?

We have been interested to verify if the fluoxetine is able to reverse the reduced hippocampal neurogenesis in PRS rats because already several antidepressants increase both cell proliferation and neurogenesis in dentate gyrus of the adult hippocampus.

5. RESULTS

The prenatal environment exerts profound influences on the development of organism, inducing changes that extend from early to later life. Using PRS, an animal model that induces long-lasting alterations, several of which are in common with depressed patients, we have been studied circadian rhythms, hippocampal plasticity, and new antidepressants in adult offspring. Taken together our results indicate that PRS induces abnormal circadian and sleep functions as well as radical changes in brain neuroplasticity. On the other hand the antidepressants, that we tested, are capable to reverse the PRS abnormalities.

In the first chapter the data about the PRS influences on circadian rhythms and on hippocampal neuroplasticity will have shown, then, data concerning the efficacy of new therapeutic strategies to keep down the PRS dysfunctions will follow in the second chapter.

5.1 Effects of prenatal restraint stress on circadian rhythms and on hippocampal plasticity: behavioural and neurobiological alterations that can be related to depression symptoms.

Depression is known to alter pivotal function, such a mood, cognition, or psychomotor activity and it is frequently associated with structural changes in the brain and decreases neuroplasticity. Reduced hippocampal cell volume has been observed in patients with stress-related depression (Bremer et al., 2000; Sheline 2003) in both magnetic resonance imaging and pos-mortem studies, compared with normal individuals. Such reduction in hippocampal volume might be due to glial and neuronal atrophy or loss, which is related in part to increases in corticosteroids.

Another hallmark of human depression is an alteration in the sleep/wake cycle, including a shortened REM sleep latency during the first part of night, increased sleep fragmentation, and a decrease in the amount of slow-wave sleep. Sleep is an

important factor of mammalian homeostasis and it is necessary for survival. It is a dynamic process that involves several brain structures and all physiological system of organism.

In recent years, some studies have showed how the production of new cells and their development into neurons is affected by sleep and by sleep loss; other studies have addressed specific changes in sleep/wake architecture in rodents following modulation of metabotropic glutamate receptors activity.

mGluRs are reunite in different groups and cover several functions: they modulate neuronal excitability and synaptic plasticity through regulation of ion channels and ionotropic receptors, in addiction, they are implicated in the regulation of emotional states, anxiety-like behaviour, spatial memory (Zuena, Mairesse et al., 2008), and recent data demonstrate that activation of mGlu2/3 contributes directly or indirectly to regulation of REM sleep (Ahnaou et al., 2009).

In the following sections the data concerning the effects of prenatal manipulations on circadian rhythms and on neuroplasticity will be discussed.

a- Effects of PRS on circadian rhythms

It has been shown that circadian function and/or sleep patterns can also be reset by neurochemical or behavioural stimuli. Among the stimuli, steroids can have marked effects on the functioning of the circadian system; in fact, chronic stress in adult rats can induce alterations in circadian patterns. In particular, perinatal events seem to have complex influences on the long-term development of circadian functions.

In this section focused on locomotor activity, on sleep/wake cycle and on daily hypothalamic CRH level in controls and PRS rats.

- PRS and locomotor activity

This study will be included in the following publication in preparation.

Gender specific effect of prenatal restraint stress on the rat's circadian pattern of activity.
J. Mairesse, A. Giovine, V. Silletti, G. Van Camp, C. Cinque, P. Navarra, A. Catalani, G. Mennuni, O. Van Reeth and S. Maccari

Little is known on the effects of PRS on the functioning of the circadian system in adulthood. In order to clarifying the relationships between PRS and circadian system, we have monitored the running wheel behaviour in male adult PRS rats, first under a regular 12 hour light-dark (LD) cycle (light on at 8.00 AM), and then after an abrupt 6 hours advanced shift in the LD cycle.

PRS alters the circadian activity rhythm in PRS rats:

- inducing a significant phase advance in the circadian activity rhythm compared with control animals both in **onset** of locomotor activity (defined as the moment at which the mean intensity of activity is above 10 % of the maximum value of the animal activity and it is maintained at the same level during the following 30-60 minutes), calculated on 8 days (T-Test. * P < 0.05) (FIG.9, A-B) and in **offset** (defined as the moment at which the mean intensity of activity is below 10 % of maximum value of the animal activity and it is maintained at the same level during the following 2 hours), calculated on 8 days (T-Test. * P < 0.05) (FIG.9, A-C). Moreover, PRS increased total locomotor activity, measured as mean number of wheel revolutions per day (T-Test. * P < 0.05) (FIG.9, D-F). These changes were due to changes in the intensity of activity without any change of activity duration (alpha). In fact, the alpha (defined as the total length of time of locomotor activity, to be more precise, the time elapsing between the onset and offset of activity) is not significant different between the two animals groups (T-Test. P n.s.) (FIG.9, E);

- enhancing the time required to resynchronize the activity rhythm after an abrupt phase advance of the LD cycle, six hours (T-Test. * P < 0.05) (FIG. 10, A-B). The time to resynchronize to the new LD cycle is defined as the number of days for the animal to exhibit a regular activity for at least three following days.
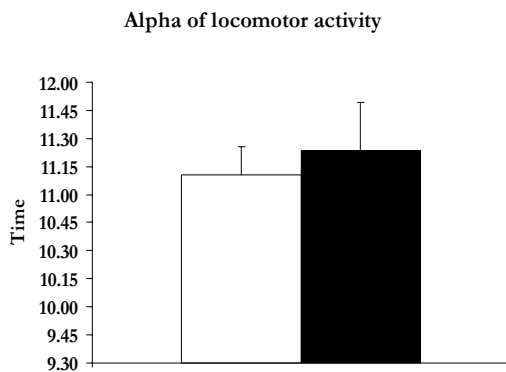
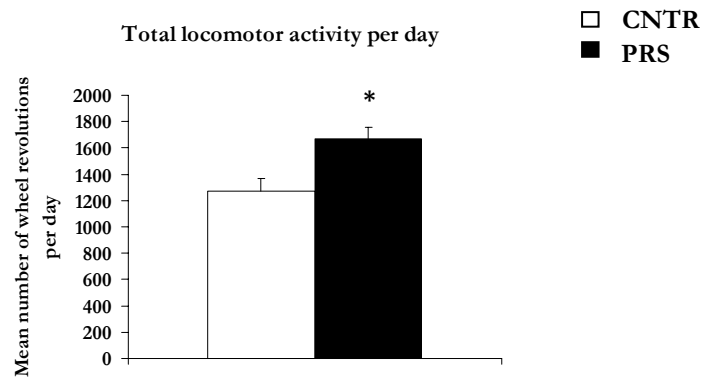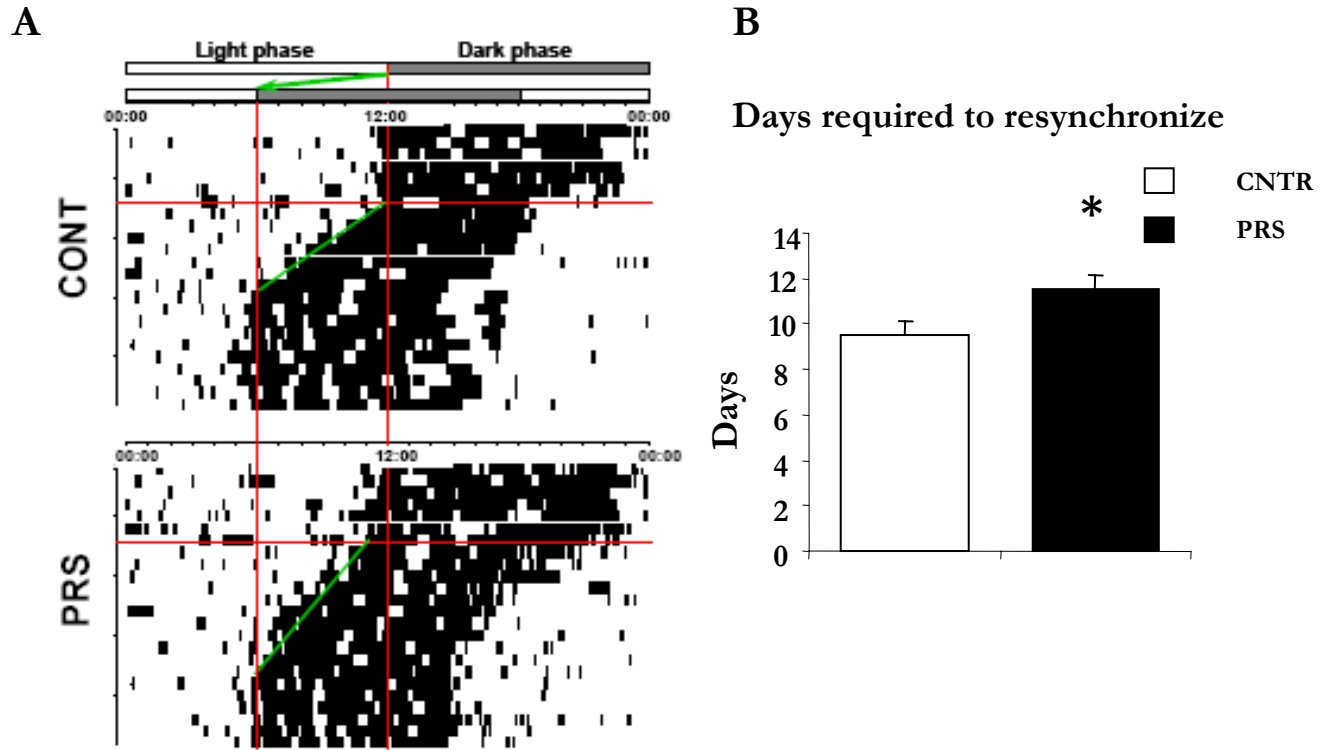**FIG. 9: Circadian fluctuations of locomotor activity after PRS.**
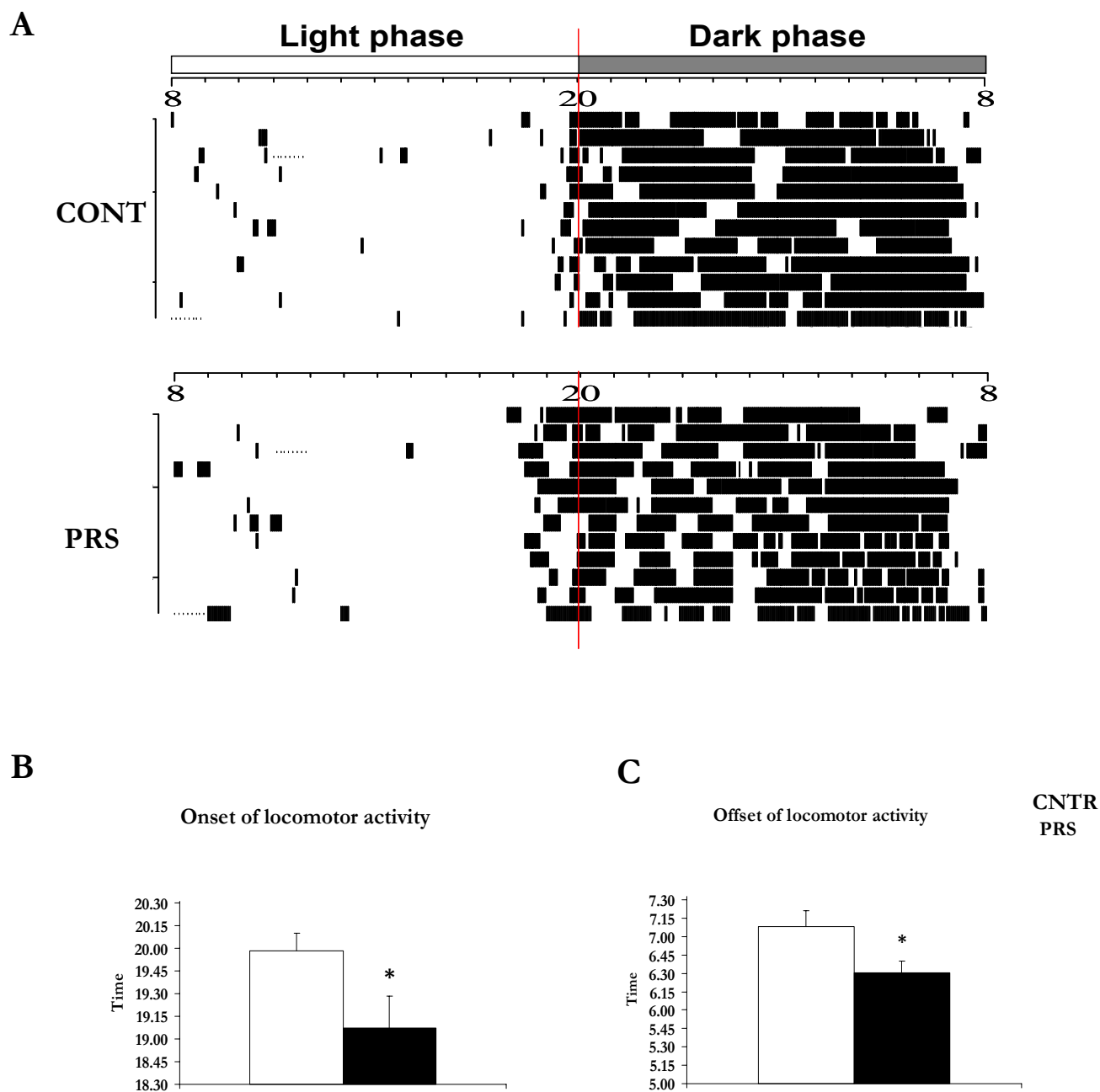
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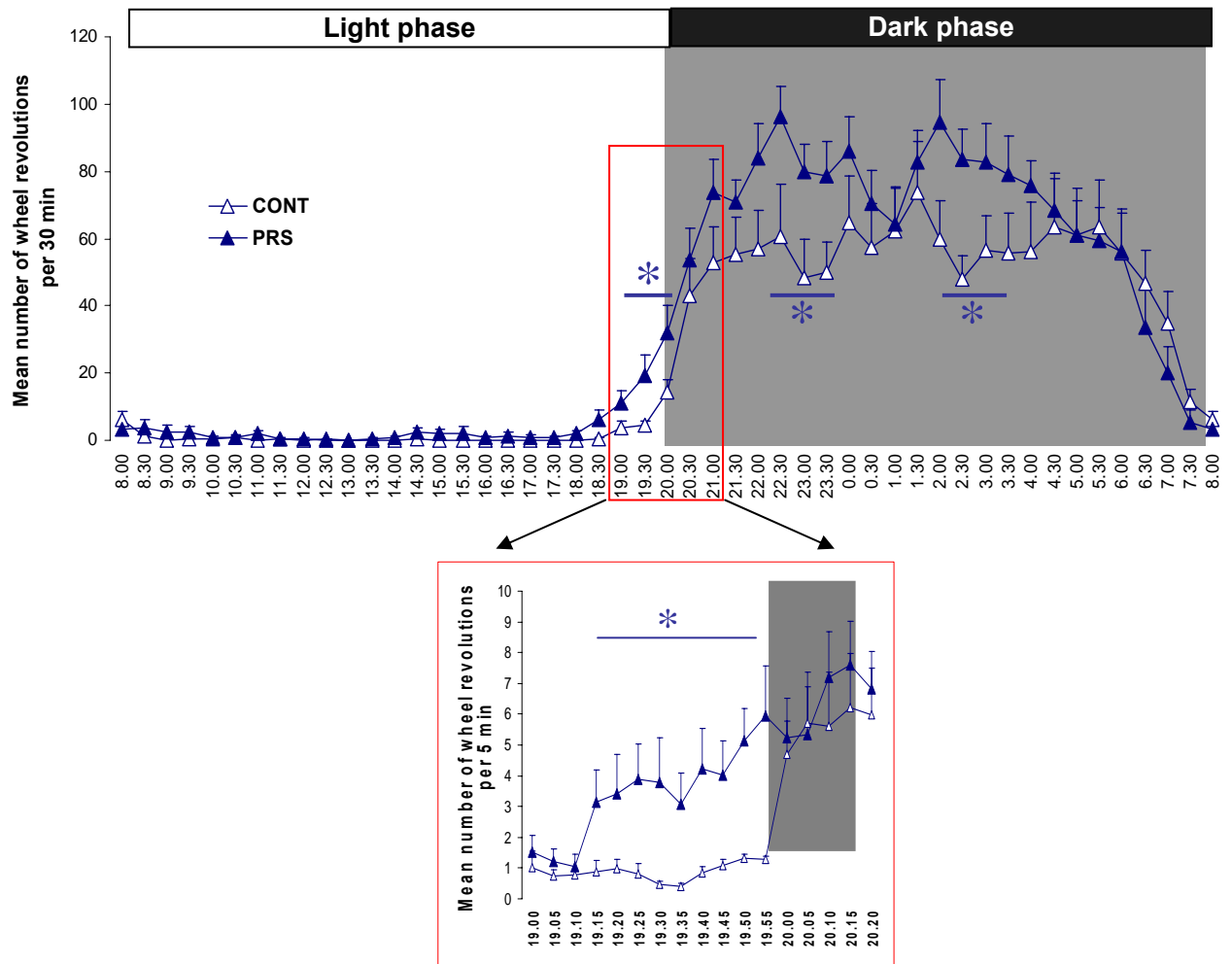
E

F

FIG.10: Locomotor activity resynchronization after PRS.

Those observations, stressing the interactions between the stress response of an organism and the functioning of its circadian system, reinforce the idea of a general homeostatic dysfunction in animals exposed to prenatal stressful events that might at least partially explain some of their abnormal hormonal/behavioural response to stress. Moreover, those data could explain inter-individual differences in human's susceptibility to shift work or other circadian-related disorders.

- PRS and sleep/wake cycle

This study will be included in the following publication in preparation.

Antidepressant drug and circadian homeostasis: chronic AG0310 administration corrects the alteration of sleep architecture induced by prenatal restraint stress in rat.

J. Mairesse, V. Silletti, A. Giovine, G. Van Camp, S. Morley Fletcher, A. Catalani, G. Mennuni, O. Van Reeth, C. Gabriel, E. Mocaër and S. Maccari
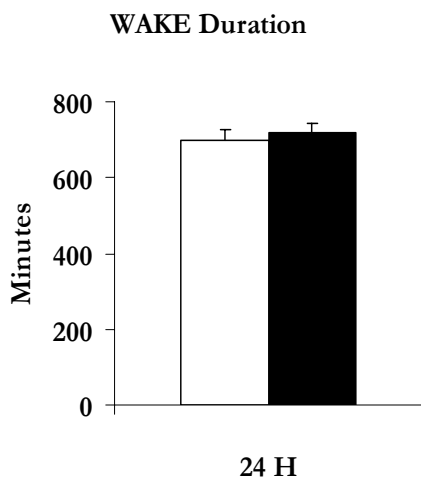
Our data show that the prenatal stress induces substantial changes in both the structure and the continuity of sleep in adult rats, triggering sleep disorders. Although the PRS does not modify the wake duration (T-Test. n.s.) (FIG.11, A-B) and the episode numbers (T- Test n.s.) (FIG.11, C), it increases rapid eye movement sleep (REM) duration (T-Test. * P<0.05) (FIG.13, A-B), as well as numbers of episodes (T-Test. * P<0.05) (FIG.13, C). In addition to the clear effects on REM sleep, PRS, also, induces an increase of the duration in slow-wave sleep (SWS) time (T-Test. * P<0.05) (FIG.12, A-B), although the number of episodes is not significantly changed (T-Test. n.s.) (FIG.12, C). Moreover, the sleep is more fragmented in PRS animals, as indexed by a larger number of episodes and a shorter duration of SWS episodes.
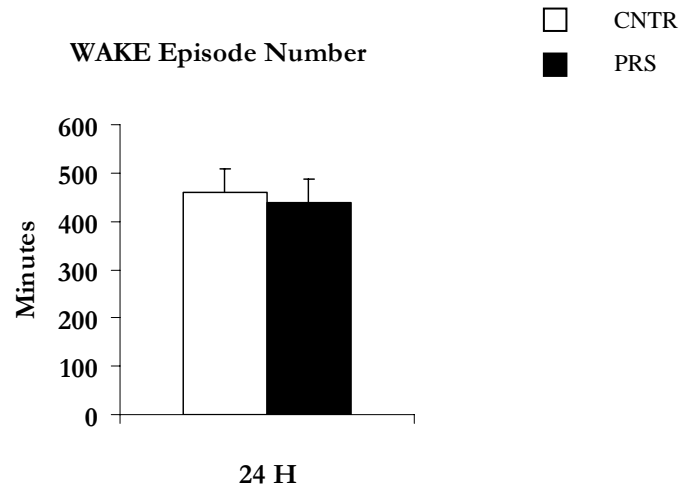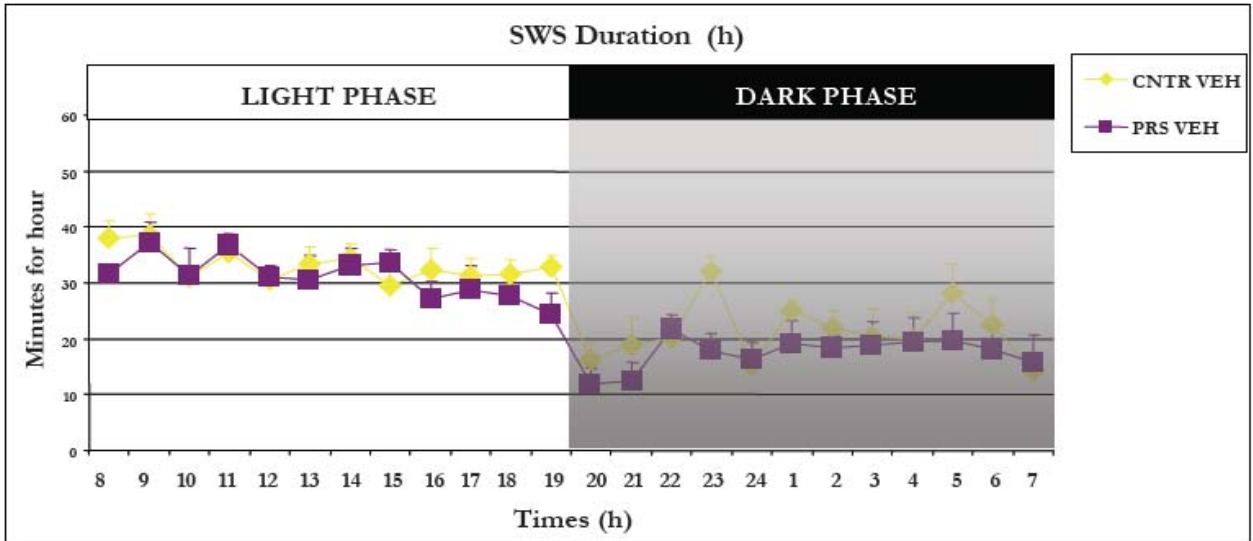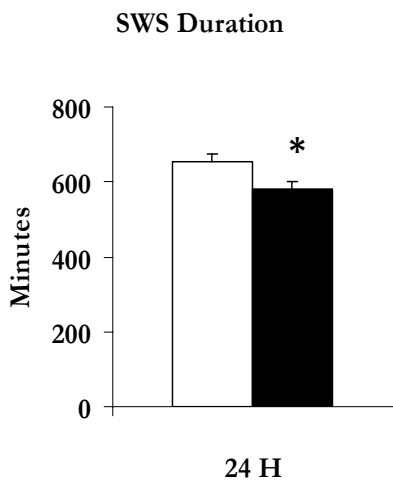
FIG. 11 Sleep/wake cycle: PRS and wake phase.

A

WAKE Duration (h)

B

WAKE Duration

24 H

C

WAKE Episode Number

24 H
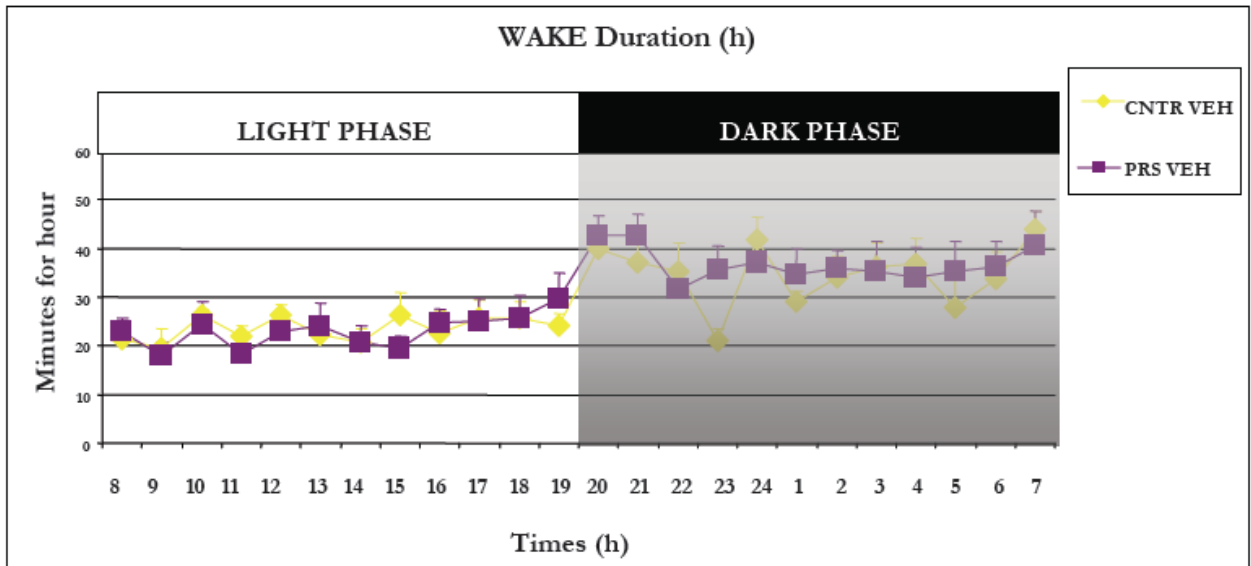
FIG. 12 Sleep/wake cycle: PRS and SWS phase.

A

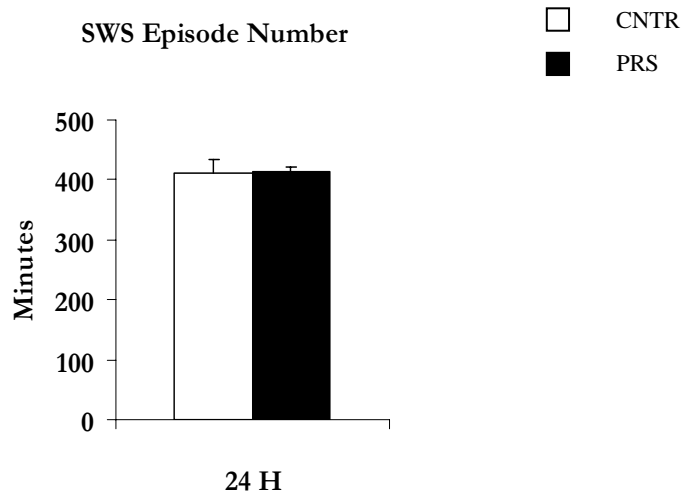

B

SWS Duration

C

SWS Episode Number

FIG. 13 Sleep/wake cycle: PRS and REM phase.

A

B

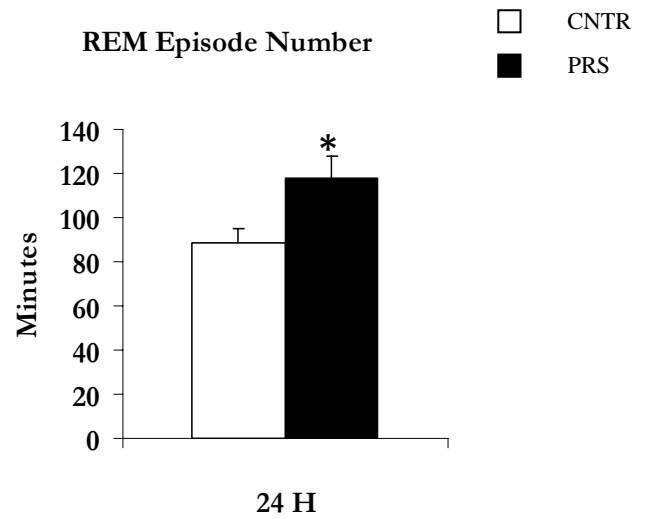**REM Duration**

24 H

C

REM Episode Number

24 H
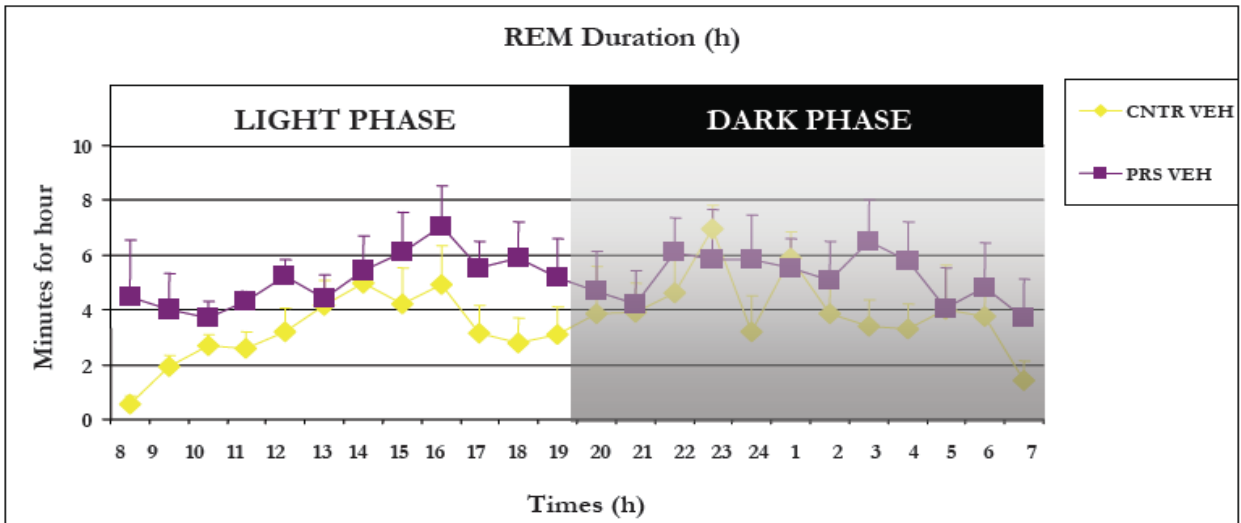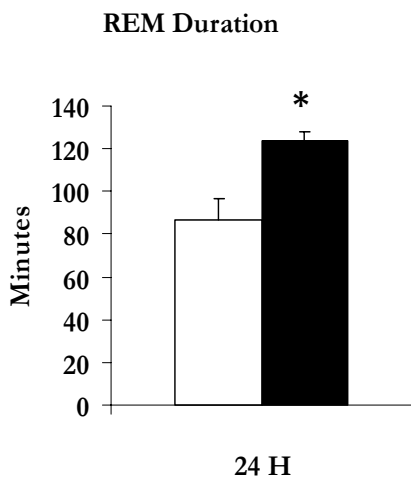
- PRS and CRH

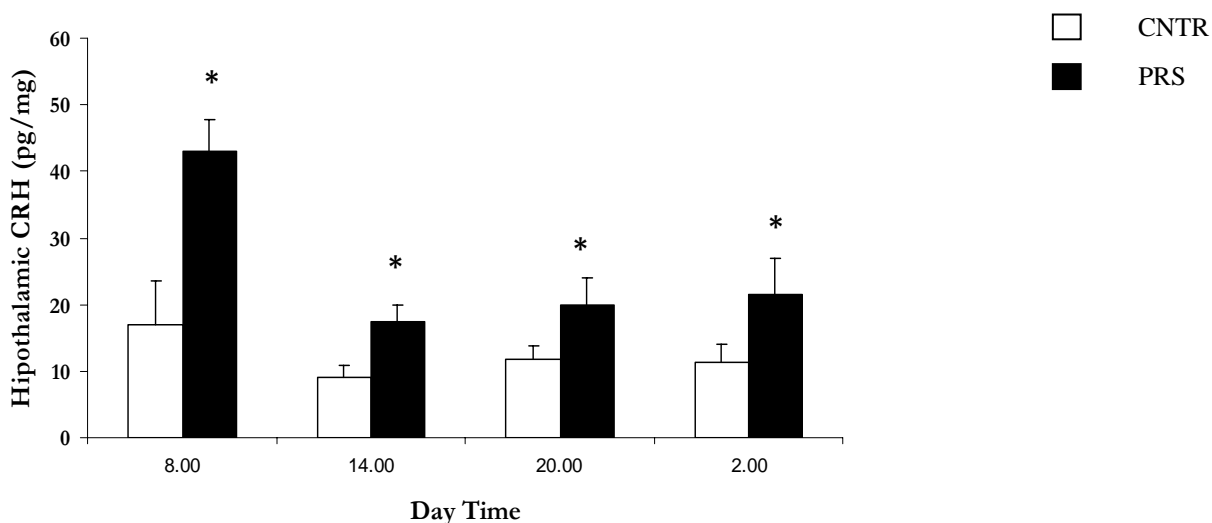This study will be included in the following publication in preparation.

Antidepressant drug and circadian homeostasis: chronic AG0310 administration corrects the alteration of sleep architecture induced by prenatal restraint stress in rat.

J. Mairesse, V. Silletti, A. Giovine, G. Van Camp, S. Morley Fletcher, A. Catalani, G. Mennuni, O. Van Reeth, C. Gabriel, E. Mocaër and S. Maccari

Sleep responds to a variety of stressors, but the precise mechanisms whereby these alterations occur are not known. Ample evidences, however, testify that CRH widely contributes to stressor-induced alterations in sleep.

The daily hypothalamic CRH level is determined to complete the role of HPA axis in circadian rhythms. Hypothalamic CRH, in control rats, shows a blunted circadian variation with a high level at the end of the night (FIG. 14). Instead, in PRS rats, hypothalamic CRH presents a more pronounced circadian rhythm levels and higher content compared to control animals at all time point (each six hour beginning 8:00 AM for all day) (T-Test.* $P<0.05$) (FIG. 14).

FIG. 14 Circadian level of hypothalamic CRH

b- Effects of PRS on hippocampal neurogenesis

This study will be included in the following publication in preparation.

***Long-term effects of chronic Fluoxetine treatment on hippocampal neurogenesis in PRS rats.***

S Morley-Fletcher, A Giovine, J Mairesse, N Haji, S Maccari

The dentate gyrus of the hippocampal formation is a site of continuous neurogenesis during adulthood in mammalian, including humans. Generated in the subgranular zone of the dentate gyrus, a portion of newborn cells becomes neurons, then, they are functionally integrated into hippocampal circuits and are involved in certain form of hippocampal learning and memory.

In this section cell proliferation was analyzed and it was addressed by thymidine analog bromodeoxyuridine (BrdU) labeling to assess the PRS effect in adult offspring. The numbers of $BrdU^+$ cells were measured in the subgranular zone of the dentate gyrus, a niche of persistent neurogenesis in the adult brain, and in the adjacent granular cell layer.

Exposure to chronic stress during prenatal life resulted in a significant decrease in the total number of BrdU-labeled cells in the dentate gyrus of the PRS rats hippocampus compared with unstressed controls (ANOVA. * $P<0.05$) (FIG.15 A). Moreover, the neurogenesis is reduced by PRS both in dorsal (ANOVA. * $P<0.05$) (FIG. 15 B) and ventral part of hippocampus (ANOVA. * $P<0.05$) (FIG.15 C). Number of positive BrdU-labeled cells in the sub-granular zone and granule cell layer in the dentate gyrus regions was combined.

FIG. 15 Hippocampal neurogenesis after PRS

A

Whole dentate gyrus

B **C**

Dorsal dentate gyrus

Ventral dentate gyrus
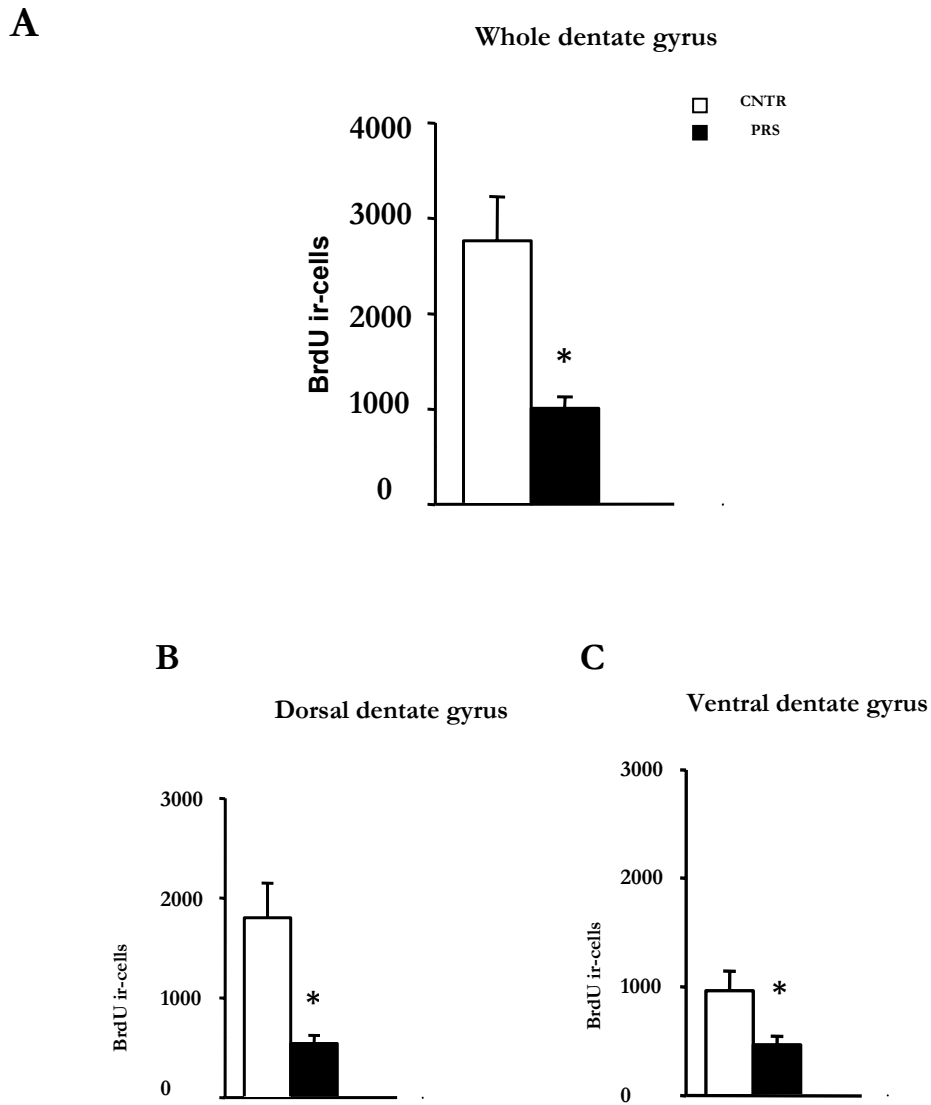
FIG.16 Immunohistochemistry of BrdU labeled cells located in the subgranular zone of dentate gyrus in A control, in B prenatal stress.

B
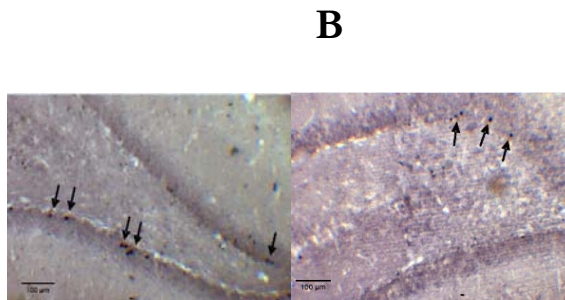
c- Effects of PRS on ontogenesis of metabotropic glutamate receptors

This study will be included in the following publication in preparation.

Ontogeny of anxiety-like behaviour in prenatally stressed rats.

Laloux C., Giovine A., Mairesse J., Garbugino L.,Van Camp G., Bouret S.G., Castro-Alvarez J.F., Branchi I., Darnaudery M., Maccari S.

Here it is reported, for the first time, the postnatal developmental profile of hippocampal metabotropic glutamate receptors expression in prenatal restraint stress rats and their respective controls at postnatal day 6, 10, 14, 22, 32, 42, 100 and at 10 month old. We examined the expression of mGlu1, mGlu5 and mGlu2/3 receptor proteins in the whole hippocampus of controls and PRS rats.

Western blot analysis of mGlu1 and mGlu5 receptors showed a 142 kDa and 130 kDa band, respectively, corresponding to the receptor monomers. No bands of higher molecular weight were detected under our experimental conditions. Blots with antibodies recognizing an epitope common to mGlu2 and mGlu3 receptors showed a 100 kDa band corresponding to the receptor monomer(s), and a higher molecular weight band (206 kDa), which may correspond to receptor dimers.

The effect of PRS on the hippocampal expression of group I and II metabotropic glutamate receptors was not homogenous, but depending of the age considered. Main effects were observed at PND 10, 22, 100 and 10 month where receptor expression was reduced by PRS.

Before weaning

Group I mGlu receptors expression is affected by PRS before weaning. mGlu1 receptors expression (not detectable at PND 6) (FIG. 17 A) is decreased in PRS hippocampus at PND 10 (T-Test. * P<0.05) (FIG. 17 B), whereas at PND 14 no more effect of PRS on hippocampal mGlu1 receptor expression is apparent (T-Test. n.s.) (FIG. 17 C). At PND 6, mGlu 5 receptor expressions tends to be decreased by PRS (T-Test. * P < 0.09) (FIG. 17 A), whereas this decrease was full

significant at PDN 10 (T-Test. *P < 0.05) (FIG. 17 B). Following the other mGlu receptors expression, at PND 14, PRS does not change the hippocampal mGlu 5 receptor expression (T-Test. n.s.) (FIG. 17 C).

On the contrary, at PND 6 (T-Test, n.s.) (FIG. 17 A), at PND10 (T-Test. n.s.) (FIG. 17 B) and at PND14 (T-Test. n.s.) (FIG. 17 C) group II mGlu receptors expression in the hippocampus is not affected by PRS.

FIG. 17 Hippocampal group I and II metabotropic glutamate receptors expression before weaning.
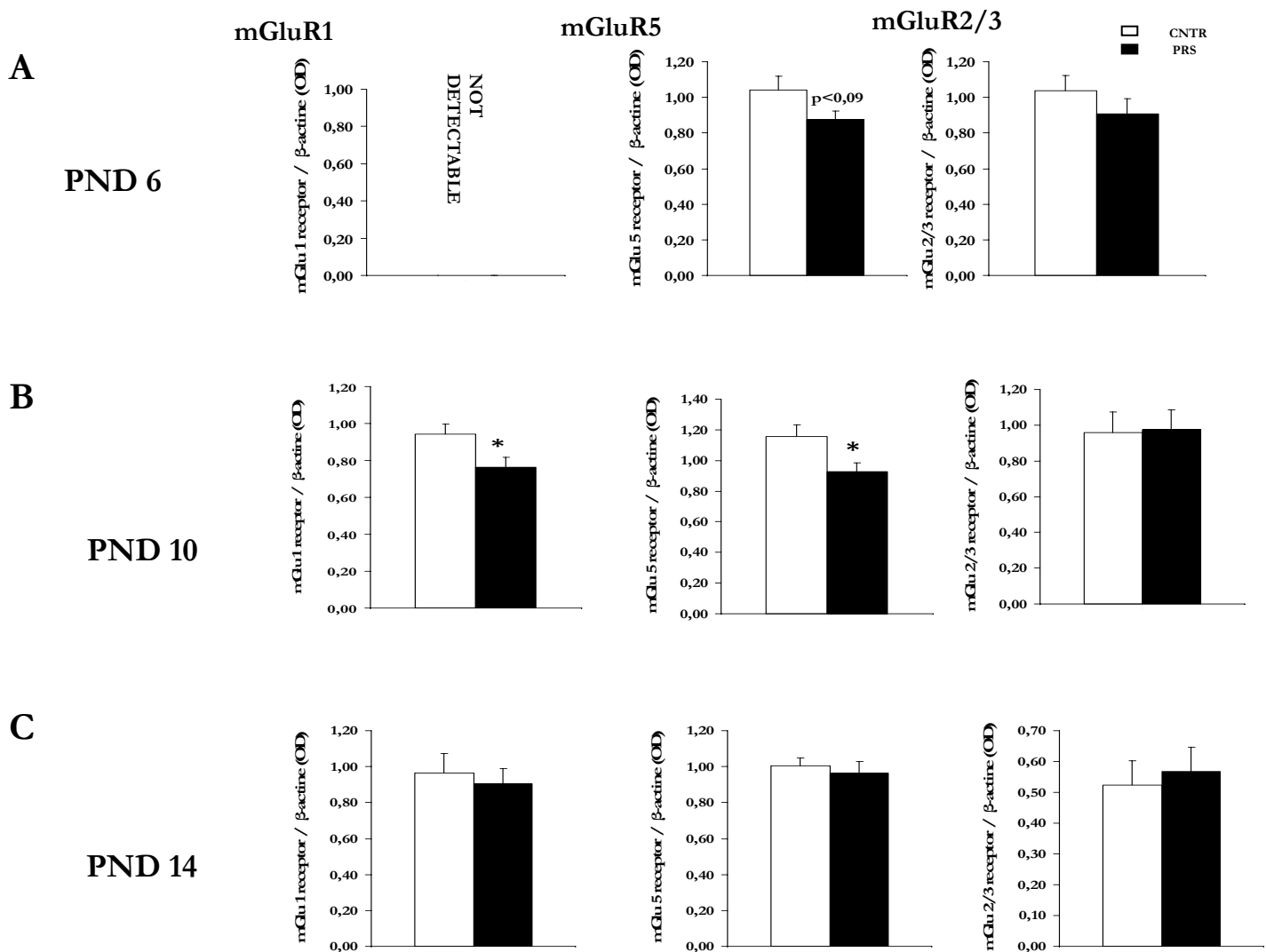
After weaning

After weaning, as before, the effect of PRS on the group I and II mGlu receptor expression is dependant of the age considered.

At PND 22, PRS induces a reduction of the hippocampal expression of mGlu 1 receptors (T-Test. * P < 0.05) (FIG. 18 A), mGlu 5 receptors (T-Test. * P < 0.009) (FIG. 18 A) and mGlu 2/3 receptors (T-Test. * P < 0.05) (FIG. 18 A).

Surprisingly, these effects of PRS disappears at postnatal day 32 and 42 where control and PRS animals show similar levels of expression for mGlu 1 receptors (T-Test. n.s.) (Respectively FIG. 18 B and FIG. 18 C), mGlu 5 (T-Test. n.s.) (Respectively FIG. 18 B and FIG. 18 C) and mGlu 2/3 receptors (T-Test. n.s.) (Respectively FIG. 18 B and FIG. 18 C).

However, in adulthood (at postnatal day 100) PRS decreases again the hippocampal expression of both mGlu5 (T-Test. * P < 0.05) (FIG. 18 D) and mGlu2/3 receptors (T-Test. * P < 0.05) (FIG. 18 D), whereas PRS does not influence mGlu 1 receptors at this age (T-Test. n.s.) (FIG. 18 D). Also in oldeness (10 months old), PRS affects the hippocampal expression of mGlu1 (T-Test. * P < 0.05) (FIG. 18 E), mGlu5 (T-Test. ** P < 0.01) (FIG. 18 E) and mGlu 2/3 receptors (T-Test. * P < 0.05) (FIG. 18 E).

FIG. 18 Hippocampal group I and II metabotropic glutamate receptors expression after weaning.
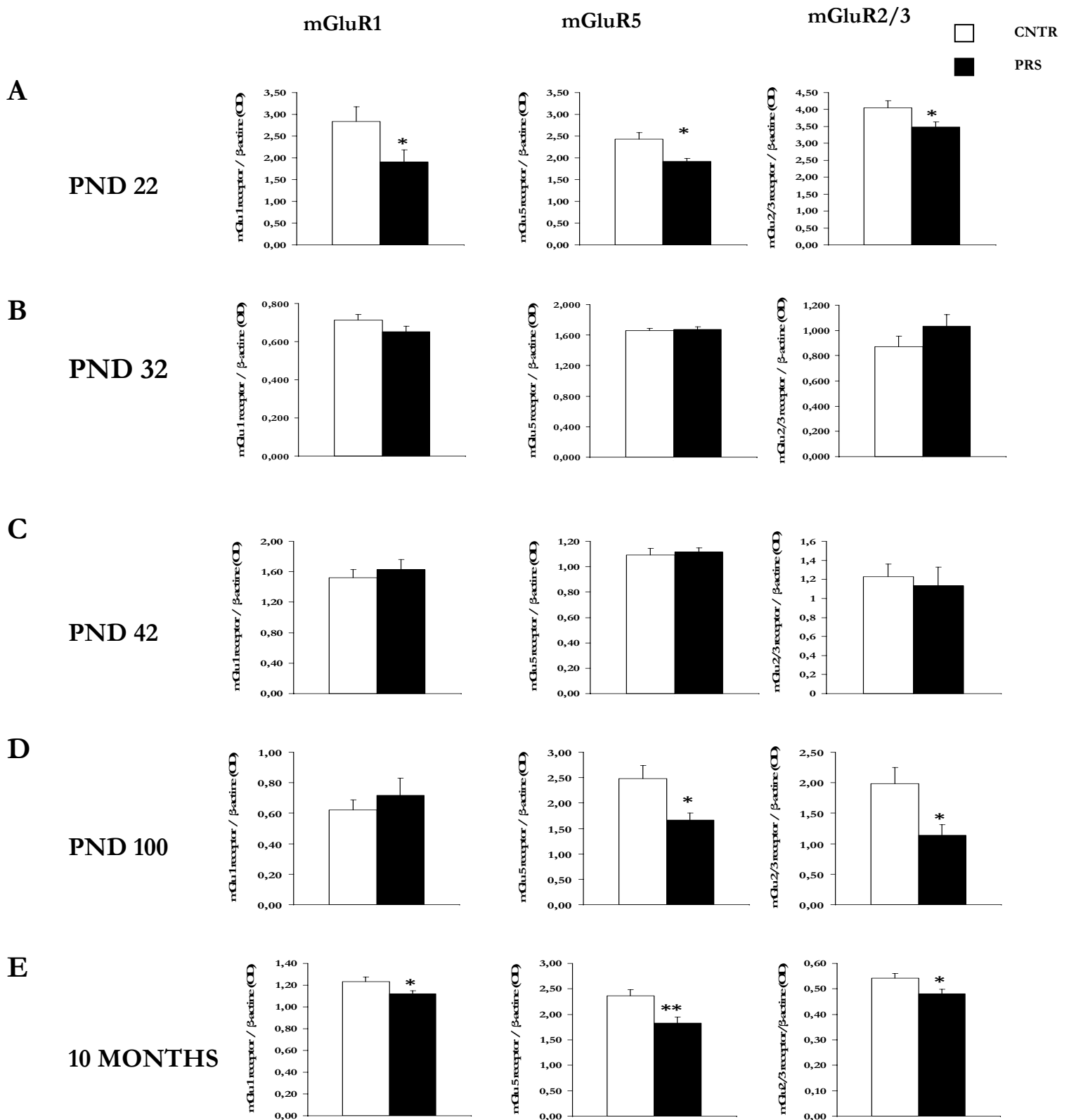
FIG. 19 Schematic view of PRS effects on mGlu receptors ontogenesis.

	mGluR 1	mGluR 5	mGluR 2/3
PND 6	=	↓	=
PND 10	↓	↓	=
PND 14	=	=	=
PND 22	↓	↓	↓
PND 32	=	=	=
PND 42	=	=	=
PND 100	=	↓	↓
10 months	↓	↓	↓

← Increased anxiety like behavior (increased ultrasonic vocalization)

← Increased anxiety like behavior (open field test)

← Cognitive impairment (Y maze) Vallee et al., 1999; Van Waes et al., 2009

← Increased anxiety like behavior (EPM test) Vallee et al., 1997; Zuena Mairesse et al., 2008; (open field) Van den Hoove et al., 2005.

mGlu5 receptors are highly express both during development and in the adult life in the hippocampus where they are involved in cells proliferation, differentiation and survival (Catania et al., 2007).

d- Proteome map of hippocampal PRS rats

This study will be included in the following publication in preparation.

Neuroplasticity in the rat model of prenatal stress: a proteomic approach.

S Morley-Fletcher, AR Zuena, A Giovine, J Marrocco, AS Vercoutter-Edouart, JC Michalski, S Maccari

Although a series of proteins in the hippocampus have been shown to be qualitatively or quantitatively deregulated by PRS, a systematic proteomic study has not been carried out so far. This study sought to generate, for the first time, a proteome map of PRS regulated proteins in the hippocampus and their implications in neuroplasticity pathways. Thus, a proteomic technique was used to search for novel proteins or signaling pathways involved in the induction of the stress response.

Data obtained concern deregulated proteins from several protein pathways and cascades including signaling, protein handling, cell proliferation and miscellaneous classes. The identification of changes in expression of protein which may serve to replace and/or restore the function of neurons lost as a result of PRS, will increase the comprehension of the mechanisms underlying the long-term effects of early life stressful events.

Hippocampal proteins were extracted and separated by 2-D electrophoresis, thus obtaining maps in which 48 spots were detectable, and 43 candidate protein spots with significantly different levels (P< 0.05) were selected. Further analysis was performed on 17 spots which did meet the minimum 1.7-fold change requirement. 8 spots were successfully identified by liquid chromatography-mass spectrometry (LCMSMS).

Biological process	Protein Name	Entry name	Accession	spot	MW Kda	pI	Mascot score	Peptides
Cell Signalling	Phytanoyl CoA hydroxylase interacting protein	PHYIP	Q56829	13	38	6.5	151	4
	F-box/LRR-repeat protein	FXL16	Q5M12	19	52	6.1	126	5
	Neuronal acetylcholine receptor alpha subunit	ACHA5	P20420	13	52	6.3	29	1
Cellular Growth /Differentiatio	Dihydropyrimidinase-like2; CRMP2	DPYL2	P47942	15	62	5.9	333	8
	Prohibitin	PHB	P67779	9	29	5.5	213	6
	LASP-1	LASP1	Q99MZ8	13	30	6.5	75	3
	CaMK2	KCC2A	P11275	19	54	6.6	61	1
Cytoskeletal protein binding	Fascin	FSCN_1	P85845	19	22	5.8	142	4
	T-complex protein 1 beta subunit	TCPB	Q5XIM9	19	57	6.01	89	2
Oligodendrocyte metabolism	Transferrin	TREF	P12346	3	76	7.1	48	1
Metabolism/energy pathways	Pyruvate kinase muscle isozyme	KPYM	P11980	20	58	6.6	322	10
	Mannose 6-phosphoisomerase	MPI	Q68FX1	10	47	5.7	180	4
	Tryptophanyl-tRNA-ligase	SYWC	Q6P7B0	19	54	6.0	149	2
	Mitochondrial import receptor subunit TOM70	TOM70	Q75Q39	17	68	7.4	126	3
	Succinate-semialdehide-dehydrogenase	SSDH	P51650	19	56	8.3	95	2
	Adenosine Kinase	ADK	Q64640	10	40	5.7	76	3
	GMP synthase (glutamine hydrolyzing); Glutamine amidotransferase	GUAA	Q4V7C6	17	77	6.2	49	2

FIG. 20: Proteins in the hippocampus identified by LC-MS/S and modified by PRS. Proteins were separated by SDS-PAGE and nano-LC-MS/MS analysis was performed after trypsin digestion as described in the Method section. Biological functions of the identified proteins are indicated according to gene ontologies obtained from the uniProtKB accession number of the Expasy server (www.Expasy.org). Exception made for two spots (10 and 13) all the spots appeared to be down-regulated by PRS between 1.8-3.1 fold. Six proteins were found to belong to the same spot (19).

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5.2 Is the chronic administration of new antidepressants AG0310 and fluoxetine able to reverse the alterations of PRS on circadian rhythms and on hippocampal plasticity?

The aim of this section is to investigate the predictive validity of the PRS rat as an animal model of depression by treating prenatally stressed animals chronically with the AG0310 or the fluoxetine and, measuring antidepressant efficacy on neurobiological and behavioural parameters.

Our previous results indicate that PRS rats showed reduced immobility in the forced swim test and upregulation of cortical 5-HT$_{1A}$ system as well as an increase of glucocorticoid receptors in response to imipramine treatment. Interestingly, imipramine had no effect on control animals (Morley-Fletcher et al., 2004).

Moreover, our last data clearly indicate that AG0310, following a chronic administration at the dose of 40mg/kg, reversed the stress-induced changes in adult neurogenesis and PSA-NCAM expression in the dentate gyrus of PRS animals (Morley-Fletcher et al., unpublished data). Interestingly, regional selectivity of the effect of stress and AG0310's action has been seen within the hippocampal formation: effects of prenatal stress and AG0310 on altered neurogenesis being observed in the ventral, but not in the dorsal dentate gyrus. Finally, AG0310 had a strong anxiolytic effect in PRS animals, as evidenced by a counteraction of the heightened anxious behaviour displayed in the elevated-plus maze (Morley-Fletcher, under revision).

a- Effects of chronic treatment with AG0310 on locomotor activity

This study will be included in the following publication in preparation

Gender specific effect of prenatal restraint stress on the rat's circadian pattern of activity.
J. Mairesse, A. Giovine, V. Silletti, G. Van Camp, C. Cinque, P. Navarra, A. Catalani, G. Mennuni, O. Van Reeth and S. Maccari

Five weeks of AG0310 oral administration (2000 ppm in dust food, corresponding to 40mg/Kg/day) abolish the difference previously observed between control and PRS rats considering the onset (T-Test. n.s.) (FIG 21 B) and the offset of locomotor activity (T-Test. n.s.) (FIG 21 C). Whereas AG0310 treatment does not modify the time of the activity onset considering both PRS (T-Test. n.s.) and control (T-Test. n.s.) (FIG 21 B) rats, it induces an anticipation of the activity offset in both groups [PRS (T-Test. *** P<0.001); (CNTR T-Test. *** P<0.001)] (FIG 21 C). In addiction, the chronic treatment with AG0310 reverses the effect of PRS on total locomotor activity, in fact, in two groups the total activity is similar (T-Test. n.s.) (FIG 21 A). About the alpha there is not significant difference between PRS and control rats after antidepressant treatment (T-Test. n.s.) (FIG 21 D). But, the AG0310 provokes in two groups of rats a decrease of alpha.

FIG. 21 Circadian fluctuations of locomotor activity after PRS and AG0310 treatment.
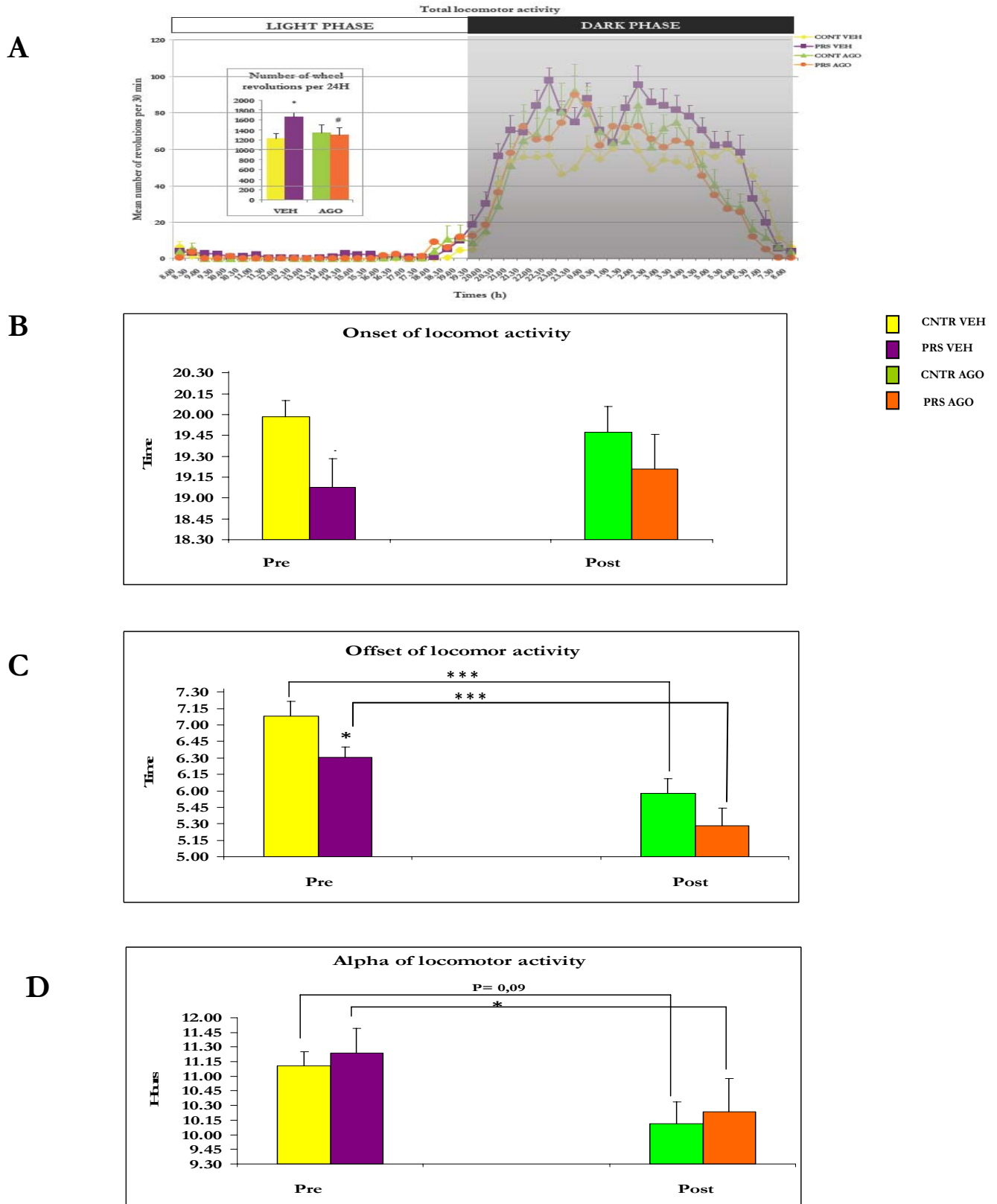
b- Effects of chronic treatment with AG0310 on sleep/wake cycle

Antidepressant drug and circadian homeostasis: chronic AG0310 administration corrects the alteration of sleep architecture induced by prenatal restraint stress in rat.

J. Mairesse, V. Silletti, A. Giovine, G. Van Camp, S. Morley Fletcher, A. Catalani, G. Mennuni, O. Van Reeth, C. Gabriel, E. Mocaër and S. Maccari

After four weeks of treatment with AG0310 (2000 ppm in dust food, corresponding to 40mg/Kg/day) the alterations on sleep/wake cycle caused by PRS disappear; while, the parameters not influenced by PRS (wake duration(FIG. 22 A-B); wake number of episodes (FIG. 22 C); SWS number of episodes (FIG. 23 C)), do not take advantage of antidepressant treatment. On the other hand, the duration of slow-wake sleep of PRS animals returns to normal level, reaching the same point of controls rats (T-Test. * $P<0.05$) (FIG. 23 A-B). In the matter of REM sleep, the AG0310 is able to reverse the changes on duration (T-Test. * $P<0.05$) (FIG. 24 A-B) and number of episodes (T-Test. * $P<0.05$) (FIG. 24 C) induced by PRS.

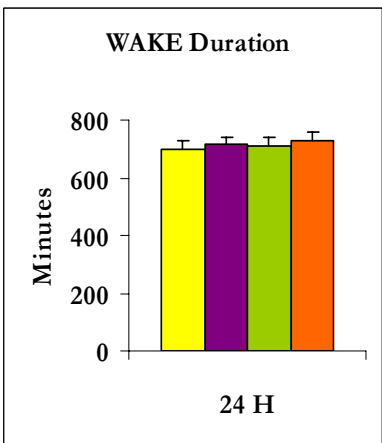To conclude, it is possible to affirm that a chronic treatment with AG0310 reverse in significant manner the PRS alterations in adult offspring.

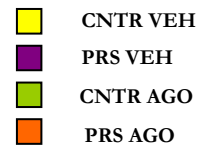**FIG. 22 Sleep/wake cycle. PRS and AG0310 treatment: wake phase.**
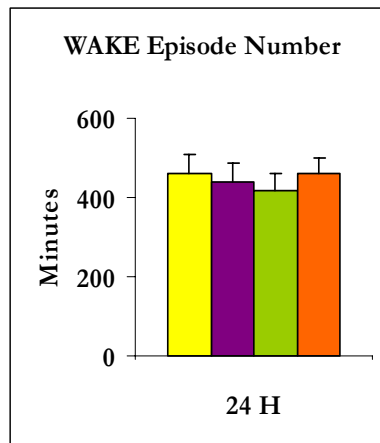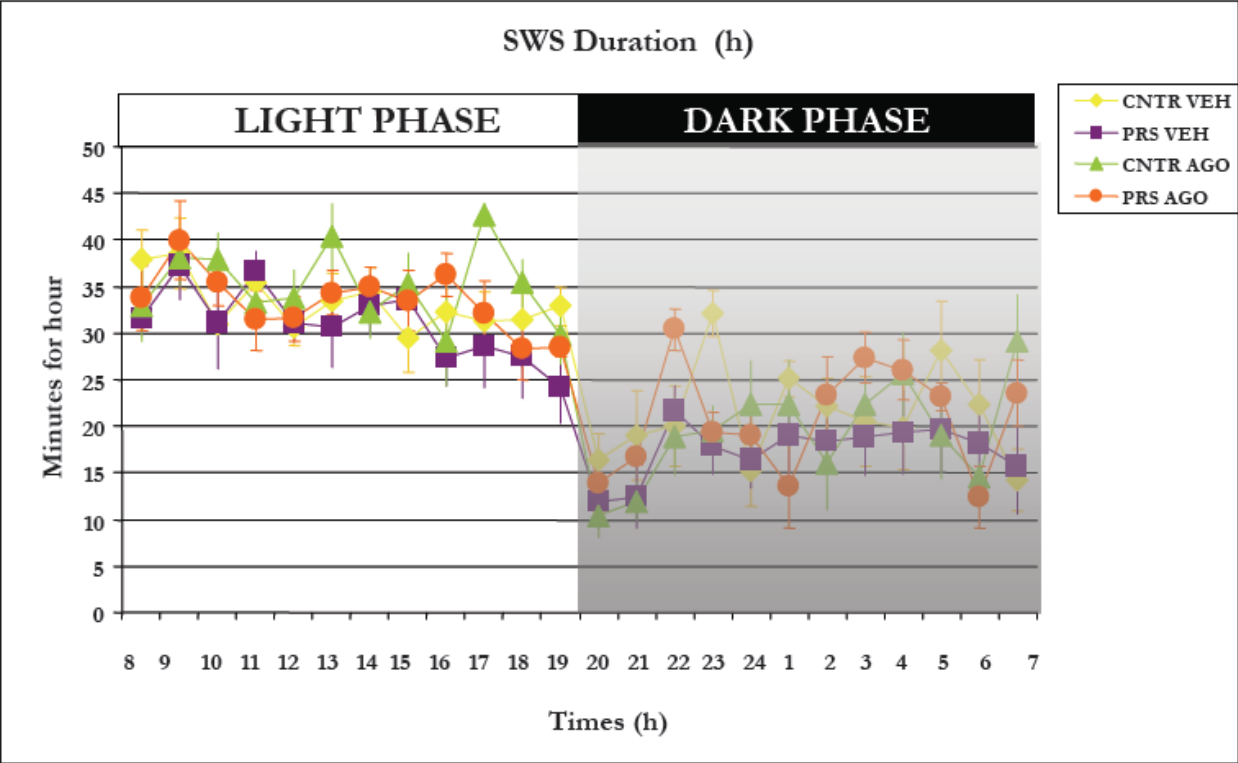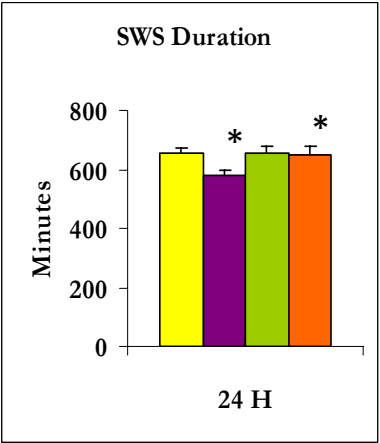
A
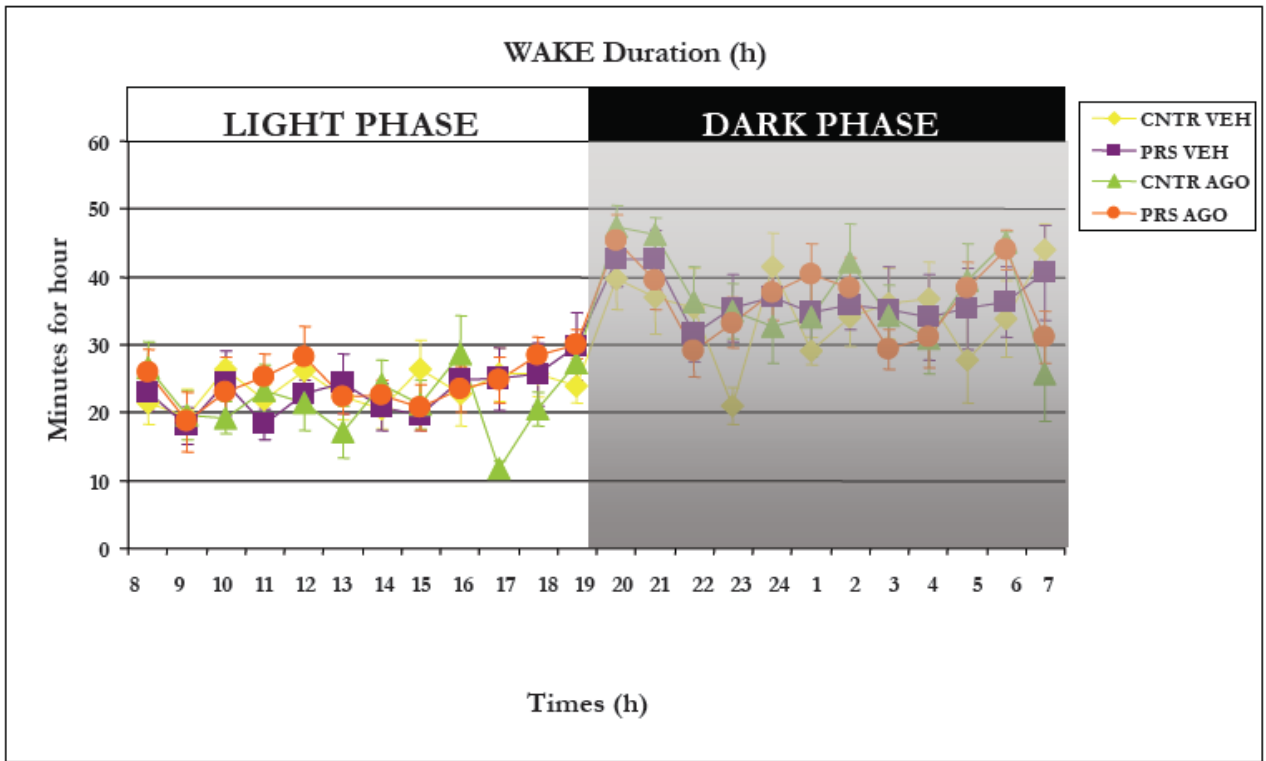
B

C

FIG. 23 Sleep/wake cycle. PRS and AG0310 treatment: SWS phase.

A

B

C
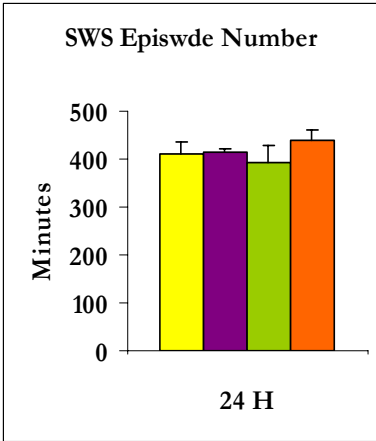
FIG. 24 Sleep/wake cycle. PRS and AG0310 treatment: REM phase.

A
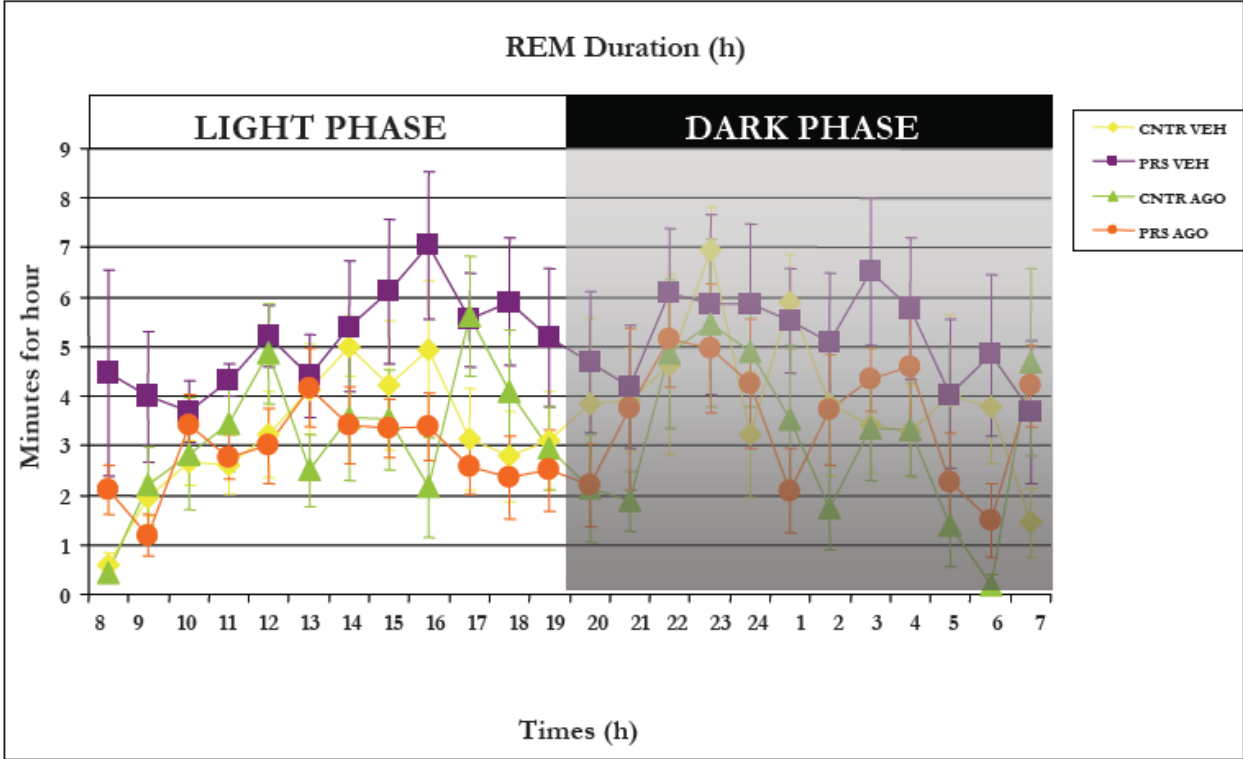
B
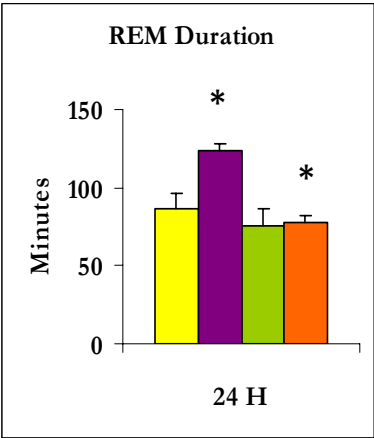
C
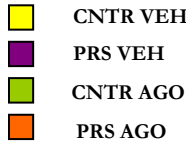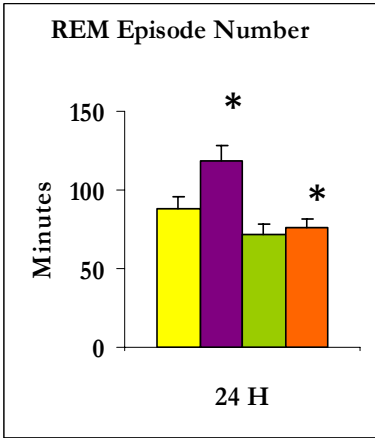
c- Effects of chronic fluoxetine treatment on PRS-induced decrease in neurogenesis

This study will be included in the following publication in preparation.

Long-term effects of chronic Fluoxetine treatment on hippocampal neurogenesis in PRS rats.

S Morley-Fletcher, A Giovine, J Mairesse, N Haji, S Maccari

As shown, PRS induced significantly decrease of hippocampal neurogenesis (BrdU-labeled cells), both in dorsal region (FIG. 25 B) and in ventral region (FIG. 25 C). After four weeks of fluoxetine treatment, the BrdU-positive cells in dentate gyrus of PRS rats increased relative to controls significantly in whole dentate gyrus (ANOVA. # P<0.05) (FIG. 25 A), and in dorsal part (ANOVA. # P<0.05) (FIG. 25 B); in contrast no significant differences were observed in ventral part (ANOVA. n.s.) (FIG. 25 C), although there wass a robust tendency to significancy. Fluoxetine not have any significant effects on no-stressed (controls) rats. Number of positive BrdU-labeled cells in the sub-granular zone and granule cell layer in the dentate gyrus regions was combined.

FIG. 25 Hippocampal neurogenesis: PRS and AG0310 treatment.

Whole dentate gyrus

A

B

Dorsal dentate gyrus

C

Ventral dentate gyrus
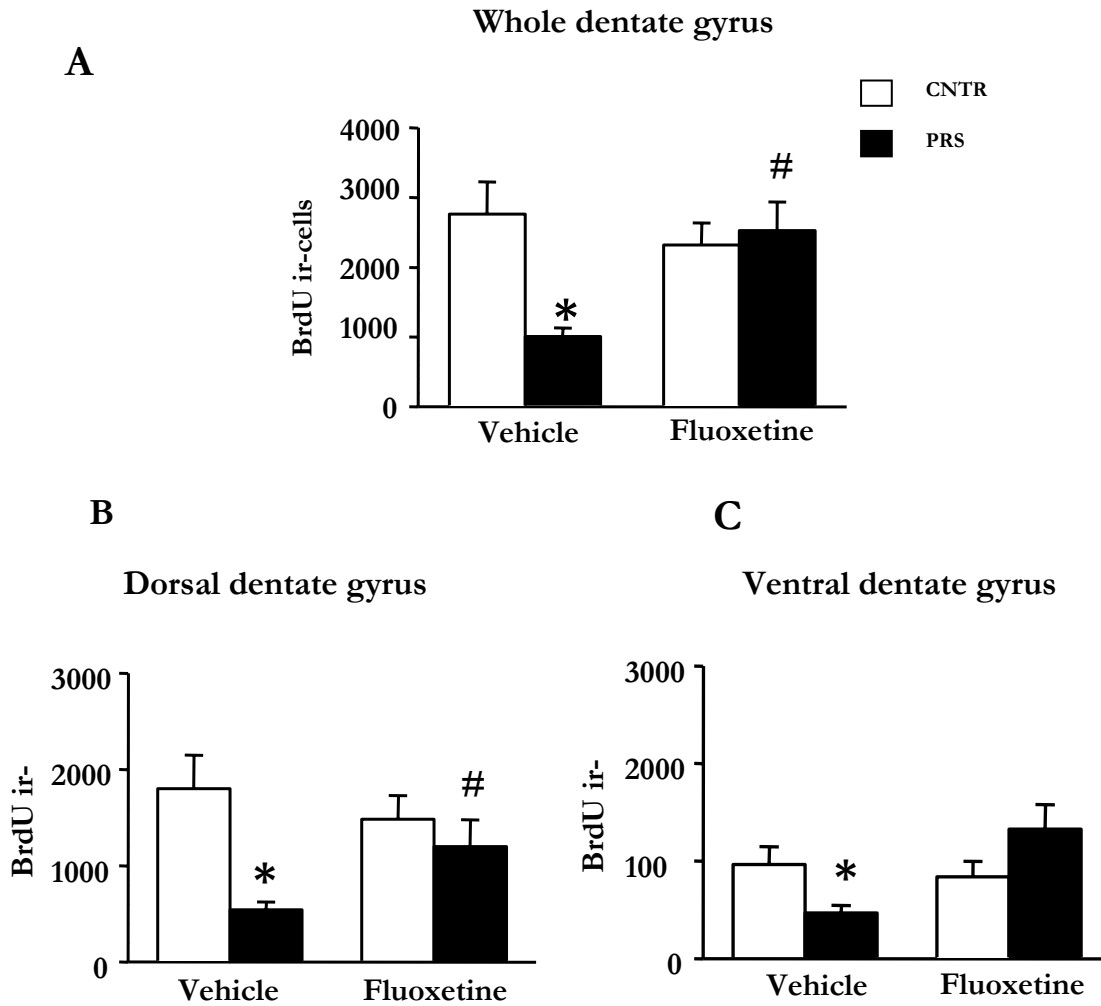
FIG. 26 Immunohistochemistry of BrdU labeled cells located in the subgranular zone of dentate gyrus in A control, in B PRS and in C PRS+fluoxetine.

A   **B**   **C**
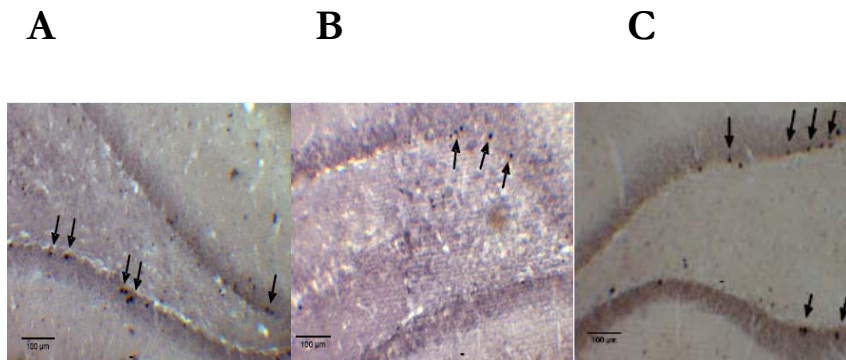
6. DISCUSSION

This thesis had the aim to assess the effects of stress hormone alterations, on circadian rhythms and on hippocampal neuroplasticity, during foetal life, period in which important developmental processes take place in the central nervous system.

Our results show that the early manipulations modifie both the parameters considered. We have been, also, interested in investigating the efficacy of two different molecules with antidepressant properties, AG0310 and fluoxetine, in reversing the alterations induced by maternal stress. Both the pharmachological treatments have proven to be effective in keeping down abnormalities at the behavioural and neurochemical levels.

The animal model of early life experience that has been adopted is the prenatal restraint stress (Maccari et al., 1995), in which the dam, during the pregnancy, is repeatedly submitted to stressful conditions. In this model a restraint stress repeated in the last days of pregnancy induces hyperactivation of HPA axis responses associated with an increase in plasmatic levels of corticosterone in offspring and other behavioural, neuroendocrine, physiological changes that have long-lasting effects on the organism. The restoration of PRS neuro-behavioural abnormalities by the chronic antidepressants treatments provides evidence of the validity of the PRS as a useful tool for studying several symptoms of mood and sleep disorders.

The alterations found in sleep/wake architecture after prenatal stress, in adult offspring, are very interesting, considering that the sleep is necessary for survival and it is a physiological event of capital importance, since, during the sleep, the recover of fundamental functions of organism takes place and memory consolidation processes are realized (Hartmann, 1973; Karni et al., 1995; Maquet, 2001). The changes in sleep induced by PRS are well linked with decrease of hippocampal neurogenesis, found from us in PRS rats, since, hippocampal

neurogenesis is reduced by sleep fragmentation in the adult rats (Guzman-Marin et al., 2007; Meerlo et al., 2009).

Following the data concerning modifications induced by PRS on circadian rhythms and on hippocampal neuroplasticity and the data about the effects of chronic antidepressant treatments on PRS-induced alterations will be separately discussed.

6.1 The effects of early manipulation on circadian rhythms and on hippocampal plasticity

The circadian system plays a major physiological role to insure optimal functioning of the organism and its adaptation to the various changes in the environment. Little is known on the effects of PRS on the functioning of the circadian system in adulthood, but, our data show that circadian rhythms of PRS offspring are significantly altered.

Obtained results evidence that PRS rats compared to controls have a significant phase advances in locomotor activity relative to the entraining LD cycle. These results are in accordance with previous results that have showed that the circadian rhythm of corticosterone secretion in PRS rats is significantly advanced like locomotor activity (Koehl et al., 1999). Moreover, the locomotor activity of PRS rats is increased and its pattern is significantly more erratic and fragmented, and, when subjected to an abrupt shift in LD cycle, PRS rats resynchronize their activity rhythm to the new LD cycle slower than control rats. Those observations, stressing the interactions between the stress response of an organism and the functioning of its circadian system, reinforce the idea of a general homeostatic dysfunction in animals exposed to prenatal stressful events.

In view of those changes in circadian rhythmicity we have investigated the effects of PRS on sleep/wake cycle. Our results demonstrate that the PRS can

produce long-term and selective changes in both structures and continuity of sleep, in fact, sleep of PRS in respect to controls rats is more fragmented and the NREM and REM sleep are modified. The episodes number of NREM sleep decreases, while the duration and the episodes number of REM sleep increase. Thus, the increase of REM sleep goes at the expense of NREM sleep duration, which is a most refreshing. In fact, the two last stages of NREM sleep are the most refreshing: the cardiac frequency, the blood pressure, the glucose metabolism, the sympathetic tone are reduced; instead, the anabolic hormones as growth hormone, prolactin, luteinizing hormone, testosterone are increased, supporting muscle growth; while the catabolic hormones, as cortisol, are reduced (Van Cauter et al., 2008). Consequently, the animals exposed to stressuful events during early life have sleep disorders that do not permit them to restore.

PRS sleep alterations are in accordance with previous studies that have observed that adult PRS rats present a disturbed HPA axis feedback characterized by a prolonged HPA activation after stress exposure and a reduction of the hippocampal GR and MR content (Maccari et al., 1995). These changes can be provoked by reduced placental protective function resulting from less important expression and activity of the 11β-HSD2 that permit to the maternal GCs, highly secreted during the restraint stress, to reach the foetus (Mairesse et al., 2007). The HPA axis is notoriously linked to circadian rhythms (Van Reeth et al., 1991; Koehl et al., 1997; Dugovic et al, 1999; Koehl et al., 1999), and because of its hyperactivation the PRS animals have worsen ability of adaptation to the changes both in external daily environment (our data showed that PRS rats required more day to resynchronize their locomotor activity after advance of phase of DL cycle) and in internal physiological processes. Both in humans and rats the activity period is characterized by increase of glucocorticoid level, instead, during the sleep the HPA axis is

quiescent. Thus the normal architecture of sleep is closely related to a good activity of axis.

In view of these results on circadian rhythms and of their link with previous data on HPA axis alterations in PRS offspring we have examined the circadian level of hypothalamic CRH. In addition to the well-established neurohormonal role of CRH in activating the HPA axis (Vale et al., 1981; Rivier et al., 1982; Owens and Nemeroff, 1991), this peptide, has relevant role in mediating central nervous system responses to stressors (Koob and Bloom, 1985; Heinrichs et al., 1995; Koob, 1999; Koob and Heinrichs, 1999; Bakshi and Kalin, 2000; Deussing and Wurst, 2005). Stress induces arousal (Chrousos, 1998) and CRH has been implicated in stress-induced alterations in sleep (Gonzalez and Valatx, 1998; Chang and Opp, 2002), particularly in the control of rapid eye movement sleep (Gonzalez and Valatx, 1997). For example, administration of CRH antagonists have been reported to eliminate REM rebound after immobilization stress (Gonzalez and Valatx, 1997) and to decrease REM rebound after sleep deprivation (Gonzalez and Valatx, 1998). Our data are in accordance with these results, in fact, PRS animals show more pronounced circadian rhythm levels and higher content of CRH compared to controls animals at all time point, and, in addiction, their REM sleep increase in respect to controls. Thus, an increase in REM duration and episode numbers corresponds to increase in levels of CRH expression.

In addiction to sleep alterations our results show that exposure a chronic stress during prenatal life result in a significant decrease in hippocampal neurogenesis, in whole dentate gyrus and in both dorsal and ventral part of it. These data are appealing considering that are in accordance with the results of other authors and with our previous findings. Fist of all PRS is able to affect the two hippocampal regions that can be differentiated on the basis of their protein expression and theirs behavioural implications. For example, in control rats, NMDA and AMPA receptor

subunits are differentially expressed in the dorsal and ventral hippocampus (Pandis et al., 2006). At behavioural level, the dorsal hippocampus can be mainly related to learning and memory process, whereas ventral hippocampus can be primarily implicated in emotional processing as the anxiety modulation (Kjelstrup et al., 2002). Second these data are relevant considering that the hippocampus is one of the cerebral structures exerting the most important inhibitory feedback on the HPA axis (for review, Herman et al., 2005), on the other hand adult neurogenesis is regulated in part by adrenal steroids therefore by HPA axis that is hyperactivated in PRS animals. Third the decrease in hippocampal neurogenesis in experimental group can be in part caused by PRS alterations in circadian rhythms. In fact, recent data report that long term voluntary running days induce a strong down-regulation of progenitor proliferation rate to approximately 50% of non-running controls; instead short term voluntary running days potently stimulate neurogenesis. These former findings were paralleled by a gradual activation of the HPA axis and the opioid system (Droste et al., 2003 Naylor et al., 2005; Lou et al., 2008). Furthermore, by decreasing or modulating the daily running distance of long-term running animals, the HPA axis activation is prevented and a return to normal proliferation levels is found (Naylor et al., 2005; Lou et al., 2008). Hence, prolonged running can develop into a stressor, overruling the positive effects of exercise on neurogenesis, and may even induce dependency-like behaviour (Droste et al., 2003). This suggests that positive stimuli for neurogenesis can only be effective when HPA axis activation is minimal. Moreover, several studies demonstrate that sleep fragmentation reduces hippocampal neurogenesis (Tung et al., 2005; Guzman-Marin et al., 2007). Thus, the sleep alterations of PRS rats would in part be the cause of their decrease in hippocampal neurogenesis. However, the mechanisms by which sleep affects different aspects of neurogenesis are unknown. The fact that cell proliferation does not appear to be diminished by short sleep deprivation of less

than a day (Roman et al., 2005; Van der Borght et al., 2006; Guzman-Marin et al., 2008), and that the findings that reduced proliferation after prolonged sleep deprivation does not normalize after 8 h of recovery sleep (Tung et al., 2005), seem to suggest that the relationship between sleep and neurogenesis is indirect. In other words, sleep may not promote cell proliferation and maturation directly but, instead, sleep may be essential for normal functioning of other processes and systems that, in turn, regulate neurogenesis. Prolonged sleep deprivation might affect these other processes and by that have cumulative adverse effects that gradually diminish cell proliferation and neurogenesis over the course of several days.

The sleep alterations and the reduced neurogenesis in PRS rats are in accordance with alterations in learning and memory abilities of these animals showed in a recent study (Van Waes et al., 2009), in fact, the cognitive deficit could be in part linked a these changes induced by PRS that compromise the capability to storage informations.

In this thesis we study, also, the PRS effects on ontogenesis of metabotropic glutamate receptors. The data, that we have obtained, are in according with our findings on sleep architecture and on hippocampal neurogenesis alterations induced by PRS, previously discussed. We evidence that the effects of PRS on group I (mGluR1, mGluR5) and II mGlu (mGluR 2/3) receptor expression are dependant of age considered, but at all events PRS reduces hippocampal expression of two groups of mGlu receptors. We have noticed major effects before weaning at PND 10, immediately after weaning at PND 22, in adulthood at PND 100 and in oldness at 10 months. mGluR2/3 has been shown to control the function of the HPA axis activity (Holsboer and Barden 1996; Scaccianoce et al., 2003), to exert neuroprotective effects enhancing neurogenesis (Bonanno et al., 2005), and, our data point out that PRS animals have hyperactivation of HPA axis, decrease in

neurogenesis and reduced mGlu receptor expression. Particularly interesting, the modulation of group II glutamate receptors elicits changes in rat sleep/wake architecture. The results reported in the recent studies address specific changes in sleep–wake architecture in rodents after modulation of mGlu2/3 receptor activity, in fact, mGlu2/3 receptors agonist suppress REM sleep (Feinberg et al., 2002; Ahnaou et al., 2009). Our data show that PRS animals have decrease in mGluR2/3 expression and an increase in REM sleep. The mechanisms through which mGlu2/3 receptor inhibits REM sleep are unknown. Sleep and wake are complex integrated behaviours regulated by multiple neurocircuits and neurochemical components (Zarate et al., 2004; Kugaya and Sanacora, 2005; Witkin et al., 2007).

Finally, for this section, we report, for the first time, the differences caused by PRS at the level of global protein expression in the hippocampus using a proteomic approach. In addition to neural plasticity, hippocampus plays an important role in the negative feedback regulation of the HPA axis (de Kloet et al. 1998). Both these features are permanently impaired in PRS rats (Maccari et al., 1995; Darnaudery and Maccari 2008; Zuena et al., 2008). Our study shows that exposure of pregnant female rats to a chronic stress paradigm during a critical phase of foetal brain development programmed changes in the HPA axis coupled to changes in protein expression in the hippocampus. Protein expression is significantly altered in the hippocampus of adult rats as a function of early (prenatal) stress in life. Results obtained with proteomics are relevant because this neuroproteomic approach has been able to reveal new proteins or signalling pathways involved in the regulation of neural plasticity that are modulated by PRS. Among the protein spots down-regulated by PRS several spots include proteins mediating synaptic plasticity and cell proliferation such as found 2 cytoskeleton- associated proteins like Fascin- and TCB1. PRS has led, also, to alterations in the biological protein profile (proteome) at the cytosolic and mitochondrial level, as well as proteins involved in structural

processes and metabolic and synthetic cellular maintenance. Several proteins, associated normally with outgrowth and/or maintenance of neuronal processes and with neural regeneration and axonal guidance, have been found to be modulated in the hippocampus by PRS as for example dihydropyrimidinase-related protein-2 (DRP-2, also known as CRMP2 or TOAD64), CaMK2 and LASP1. We find the differential expression of Transferrin, an oligodendrocyte-related protein. Transferrin is an iron carrier that participates in oligodendroglial cell differentiation, maturation and function (Espinosa de los Monteros et al., 1999; Martins-de-Souza et al. 2009). The main function of oligodendrocytes is the maintenance of axon myelinization in the central nervous system. The diminution or malformation of the myelin sheath can lead to a reduced propagation of nerve impulses, reducing the neuronal connectivity and triggering an immune response that can compromise tissue functioning.

These differences in protein expression may, at least in part, form the molecular basis for the effect of early-life experience on the development of the HPA responses to stress last but not least, to be involved in regulation of the neuroplasticity in the offspring that are endured throughout life. The above findings may open new opportunities for further investigations on the modulation induced in the brain by stress at the molecular level. The changes in circadian rhythms, CRH, neurogenesis, mGlu receptors induced by PRS rats, that we have previously reported, are expression of an enduring maladaptive form of neuroplasticity that underlies the anxious/depressive phenotype of PRS rats. Determination of candidate proteins, as well as the post translation modifications at proteomic level induced by PRS, will provide new insights into the epigenetic programming of the molecular machinery underlying the long-term effects of PRS on neural plasticity, brain and behaviour.

To conclude this section it is possible affirm, sure as hell, that the PRS programs at several behavioural and neuroanatomical alterations, thus, PRS animals are "genetically perturbed", since foetal stage. Each changes in biological systems of PRS rats, that we are previously discussed (HPA axis hyperactivation, decreased expression of mGlu receptors, reduction of neurogenesis, alterations in circadian rhythms), are expression of alterations in all biological systems that, submitted to allostatic overload, are completely unbalanced, uncoordinated and they are not able to interact between them to restore the homeostasis.

The PRS biobehavioural modifications, that we have been illustrated in this thesis, as altered pattern of circadian rhythms, overall impairment of the HPA axis activity and perturbation in hippocampal plasticity, can parallel to some extent indices that have been documented in a great majority of depressed patients (Rosenwasser and Wirz-Justice, 1997), suggesting, thus, that the PRS may be an animal model to study these symptoms of depression. Moreover, in contrast to other stress-related animal models of depression, the persistence of all induced abnormalities after stressor removal in PRS rats can be seen as particularly advantageous for the design and testing of therapeutic strategies in depressive disorders.

6.2 Therapeutic strategies to reverse PRS alterations

In preceding section it has been showed that the PRS produces long-lasting alterations on circadian rhythms and on hippocampal plasticity, changes that are typical symptoms of depressive disorders.

In this part we discuss our findings concerning the efficacy of two different antidepressants, AG0310 and fluoxetine, to restore previous abnormalities induced by PRS.

It is necessary identify new antidepressant therapeutic strategies for two fundamental questions. Fist of all, the long time that actual antidepressant required to be effective could contribute to high percentage of suicides among depressed patients. Second, one-third of subjects affected by depression does not respond at pharmacological treatment actually in hand.

Several work completed in our laboratory, thus, attempted to determine the effects of chronic antidepressant treatments on PRS rats at behavioural and neurochemical level. In the Porsolt forced swimming test the PRS increases the immobility behaviour and decreases the swimming. The plasmatic corticosterone levels are positively correlated with the immobility and negatively with swimming (Maccari et al., 2001). PRS rats chronic treatment with a tricyclic antidepressant such as the imipramine (3 weeks daily, 10mg/kg i.p.) decreases the immobility in this test (Morley-Fletcher et al., 2003; 2004a), and attenuates the deteriorations of the social behaviour observed in PRS rats. In order to extend this study, the effect of the treatment has been examined on other parameters. It has been observed that the imipramine alleviates the decreased GR and MR expression observed in the PRS rat hippocampus and reduces the expression of 5-HT$_{1A}$ receptors (Morley-Fletcher et al., 2004b). In addiction, a new antidepressant, AG0310, has been tested and it has been showed that it is able to reverse the PRS alteration on neurogenesis in ventral hippocampus (Morley-Fletcher et al., unpublished data).

The data on antidepressant treatments presented in this thesis, and following discussed, complete this description and reinforce the predictive validity of PRS model.

Our results show that, after chronic treatment with AG0310 (40mg/Kg/day), by virtue of its melatonin agonist, PRS alterations on locomotor activity and on sleep architecture disappear, in fact, the parameters influenced by PRS return to normal

level and it is not more possible to observe differences between PRS and control rats.

It is really appealing that this molecule is able to restore sleep architecture, considering that the sleep deregulation is a typical symptom of human depression. Moreover, it must be considered that our previous data have demonstrated that the AG0310 treatment prevented the stress-induced reduction of neurogenesis (Morley-Fletcher, unpublished) and, also, that Millan and co-workers (2005) have demonstrated that AG0310 has clearly anxiolytic effects.

It is mainly interesting to note that reversing the decreased neurogenesis, the AG0310 is able to act indirectly. In fact, this antidepressant links a melatonin agonist with 5-HT$_{2C}$ antagonist properties. Moreover, these observations highlight the existence of a strong link between the different alterations presented by PRS rats. Perhaps, through this linkage, the AG0310 treatment by acting on few target systems can restore the others impaired system reinstituting the brain plasticity.

In any case, we can affirm that AG0310 represents a new approach to treat the depression linking to efficacy a good profile of tolerance.

Finally, we have demonstrated that chronic fluoxetine treatment restores the neurogenesis in whole dentate gyrus of adult hippocampus induced by PRS. The PRS affects the serotoninergic system in hippocampus (Hayashi, 1998) and in prefrontal cortex (Morley-Fletcher et al., 2004) and 5-HT is involved in the regulation of neurogenesis (Daszuta et al., 2005). It is possible that the fluoxetine, a selective serotonin reuptake inhibitor, reverses PRS abnormalities in neurogenesis acting on serotoninergic system. But, it is, moreover, possible that this antidepressant increases indirectly the neurogenesis interacting with neurotrophic factors; Maccari and co-workers (2007) have demonstrated that the AG0310 treatment modulates the hippocampal expression of BDNF in PRS rats. In addiction, PRS reduces hippocampal expression of mGlu receptors that is linked

with a decrease of CREB phosphorylation, and AG0310 reverses these reductions (Maccari et al., 2007). Thus, the fluoxetine can act activating CREB phosphorylation that modulates, in consequence, glutamatergic system and BDNF, both implicated in neuronal plasticity.

This finding is particularly considerable: recent hypotheses sustain that antidepressant efficacy is in part due to increase in hippocampal neurogenesis, since different antidepressant classes increase both proliferation and neurogenesis in dentate gyrus of adult hippocampus. Also clinical data report that the antidepressants restor hippocampal volume in depressed patients, and it is possible hypothesize that neurogenesis recovers a great role in this process (Sheline et al., 2003). Thus, our work adds to the growing body of evidence of increased neuroplasticity as a target of the action of many antidepressants, and, further, reinforces the predictive validity of the PRS as a model of depression.

The action of the antidepressants that we have tested (AG0310 and fluoxetine) have several parallels to what is seen in the clinic: (I) full recovery at the end of treatment period (efficacy), (II), lack of significant effects in control animals (specificity), except for offset and alpha of locomotor activity and (III) chronic treatment required to reverse the behavioural and neurochemical deficits (time-course).

The lack of a significant effect of AG0310 and fluoxetine on control animals seems to contrast with previously reported data on other antidepressants' effect in non-challenged animals (Banasr et al, 2006; Malberg et al, 2000; Santarelli et al, 2003). However, differences in rearing conditions, as well as the application of a chronic injections protocol, need to be carefully considered as experimental parameters known to modulate the effect of stress and/or the action of antidepressants. Indeed, in the experiments showing an effect of AG0310 on neurogenesis under basal conditions (Banasr et al, 2006), or of other antidepressants

(Malberg et al. 2000), animals were directly obtained at the adult stage from the commercial breeder, instead of being reared since birth in the animal facilities as in the present study.

Thus, it can not be ruled out that results obtained on the so-called "non stressed" control animals could reflect the action of antidepressants on silent pre-existing alterations. These facts underlie the importance of using animal models when investigating the mechanisms of antidepressant therapy. In this regards, there is evidence that in human patients significant effects of antidepressants appear to be induced only when symptomatic targets exist (Bonne et al, 1999). It has to be noticed that sensitivity of the strain (Wistar vs Sprague-Dawley) as well as sex differences (Schmitz et al., 2002) may play an important role in shaping hippocampal sensitivity to stress.

To conclude, we can affirm that the neuro-behavioural restoration of PRS alterations by the chronic antidepressants treatments provides evidences of the validity of the PRS as a good model for studying new drugs to treat several symptoms found in mood diseases like depressive disorders.

6.3 Perspectives

Finally, we propose the continuation of this thesis work. In the light of our results on alterations induced by PRS on sleep/wake architecture: increase in REM sleep, decrease in SWS and a higher sleep fragmentation, and of results obtained previously from our group that showed that PRS has pro-inflammatory consequences on the immune system in adult rats (Vanbesien-Mailliot et al. 2007), and considering that both abnormalities are typical symptoms of human depression (Benca et al., 1992 Irwin, 1999), we want to try to study the link between sleep and immune system, using PRS model.

Actually, the knowledge on this linkage are scanty, it is yet not clear if the immune system influences the architecture and the continuity of the sleep (Moldofsky, 1994), or if the sleep, considered as state of the brain, improves the activity of the immune system (Lange et al.,. 2003). Many questions are still without answer.

However, numerous studies suggest that complex cytokine network interacts with sleep/wake cycle (Majde e Krueger, 2005) by means of neurotransmitters and hormones (Kapsimalis et al., 2005; Opp et al., 2005). Circulating concentrations of IL-1 increase at the onset of sleep (Irwin, 2001) and this cytokine with the Tumor necrosis factor are involved in regulation of NREM sleep (Kapsimalis et al., 2005). Moreover, the circulating concentrations of IL-6 show a periodicity with low values during daytime and maxima during the night (Bauer et al., 1994), and IL-4 and IL-10 inhibit sleep (Majde e Krueger, 2005).

On the other hand, during mild infectious diseases the want to sleep is increased and sleep architecture is modified, the lack of sleep increases susceptibility to infections (Toth, 1995), and, finally, sleep improves immune defence (Lange et al. 2003). These findings have fostered ideas that there is a relationship between sleep and immune system, our research project in future is to investigate the link between these two dynamic processes, that involve many brain structures and all physiological systems of an organism and that are fundamental factors of mammals homeostasis and are necessary for survival.

7. MATERIALS AND METHODS

7.1 Animals and conditions of rearing

Nulliparous female Sprague-Dawley rats, weighing approximately 250g each, were purchased from a commercial breeder (Harlan, Italy or Iffa Credo, France). Animals were kept at constant temperature (22±2°C), with a regular 12hr light/dark cycle (lights-on at 8.00 a.m.). Tap water and standard food were available *ad libitum*. For a week after arrival, females were group-housed (4 per cage) to coordinate their estrous cycle. Females were, then, housed with a sexually experienced male for seven days (the following day being designated as day 0 of gestation). From then onwards, female rats were housed individually in Plexiglas cages (30x20x15 cm). Pregnant females were randomly assigned to prenatal restraint stressed or control groups. (n= 12 per group).

7.2 Stress procedure

PRS was carried out according to our standard protocol (Maccari et al, 1995): from day 11 of pregnancy until delivery, pregnant female rats were daily subjected to three stress sessions (45 min.; starting at 09:00, 12:00 and 17:00 h), during which they were placed in transparent plastic transparent cylinders (diameter = 7 cm; length= 19 cm) and exposed to bright light (650 Lux). Offspring were weaned 21 days after birth, and only male offspring from litters containing 10 - 14 pups with a comparable number of males and females were used for the experiments. In order to minimize litter effects, a maximum of two male pups were included per litter. After weaning, male rats from each experimental group (control and PRS) were housed in groups of three and maintained under the same environmental conditions until the experiments were started. All experiments followed the rules of the European Communities Council Directive 86/609/EEC.
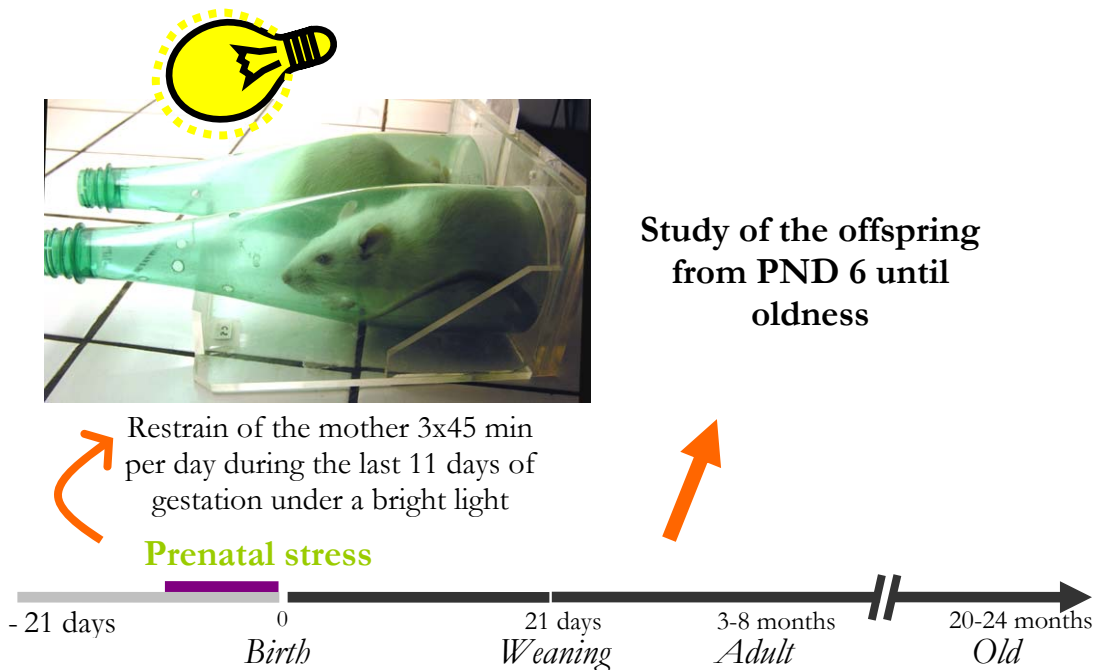
FIG.27: The prenatal stress procedure

7.3 Running wheel activity under a regular 12/12 LD cycle and abrupt phase advance

All rats (12 per groups) were 4-7 months old at the time of experiments. Rats were housed in light tight chambers equipped with continuously operating ventilating fans, and, they were placed in individual cages with a running wheel that allowed continuous recording of locomotor activity via an on-line computer (Chronobiology kit, Stanford Software System, CA, USA), under a regular 12/12 light/dark (LD) cycle (light intensity was set at 20–30 lux at cage floor level). During the course of the experiments, food and water were provided ad libitum, room temperature (22 °C±2°C) and humidity (60 %) were kept constant.
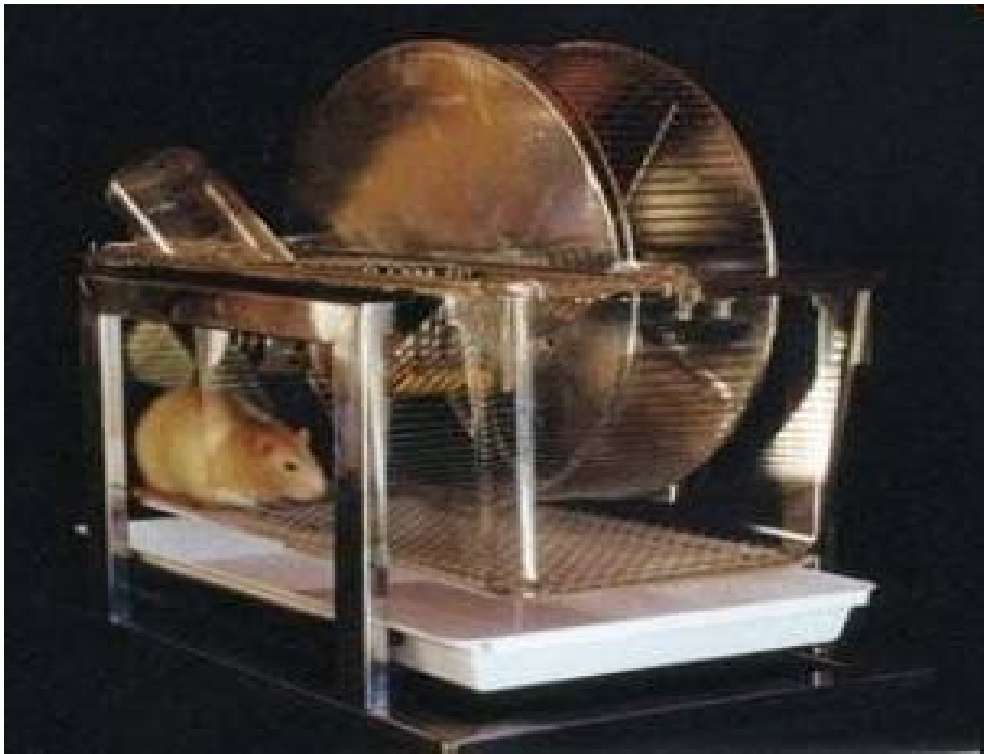
FIG. 28: Cage with a running wheel used in this experiment connected with computer to allow a continuous recording.

A first group of rats (PRS and controls) was used to analyze the rhythm of circadian activity under a regular 12/12 LD cycle, while the second group (PRS and controls) to evaluate the response to an abrupt 6 hours phase advance of the LD cycle.

After 3 weeks of adaptation to the running wheels, the rhythm of activity were individually analyzed over 10 consecutive days. The onset of activity was identified with a 5 minutes resolution and was defined as the moment at which the mean intensity of activity is above 10 % of the maximum value of the animal activity and it is maintained at the same level during the following 30-60 minutes. The reversed procedure was used for the cessation (offset) of activity, defined as the moment at

which the mean intensity of activity is below 10 % of maximum value of the animal activity and it is maintained at the same level during the following 2 hours. The time elapsing between the onset and offset of activity was defined as the total time of nocturnal activity, the peak value of activity (measured as mean number of wheel revolutions per day) and peak hour of activity (alpha, defined as the total length of time of locomotor activity, to be more precise the time elapsing between the onset and offset of activity) were directly determined on the actogram for each animal. The mean 24 h integrated activity was determined by adding minute-by-minute the number of revolutions in the wheel for each animal. The data were then plotted hour-by-hour; this represented the mean distance run by the animals.

At the end of the recording of normal activity, the rats were exposed to an abrupt 6 h advance of the LD cycle: on day zero (D0) of the experiment, lights were turned off 6 hour before than previous days and the new LD (12/12) cycle was maintained for several days. The time to resynchronize to the new LD cycle was defined as the number of days for the animals to exhibit a regular activity for at least three following days. The first day of regular activity respective to D0 was defined as the day of full resynchronization.

7.4 Electrode implantation and EEG sleep recording

All rats (10 per groups) were 3 months old at the time of implantation. The animals were implanted under deep anaesthesia (xylazine and ketamine, 1 ml/Kg i.m.), with chronic electrodes for polygraphic recordings of frontoparietal and parietoparietal electroencephalogram (EEG) and nuchal electromyogram (EMG). All electrodes were attached to a microconnector and fixed to the skull with dental cement.

FIG. **29:** Pictures of rats during and after electrodes implantation.

After surgery, the rats were individually housed in Plexiglas cages to permit the recovery, maintained under similar environmental conditions as before and left undisturbed for one week. Then, the animals were transferred in Plexiglas cylinder (30 cm diameter, 50 cm high), and, thus, they were habituated to the sleep recording procedure for the next 14 days. In their home cages and in the same room, the rats were connected with a cable to a rotating swivel allowing free movements, and EEG and EMG activities were recorded on a polygraph (Pinnacle Technology®, Laurence, Kansas USA) with an output connected to a computer for on-line spectral analysis of the EEG. Habituation consisted of two recording of 24 hours. At the end of the habituation period, sleep/wake cycle was recorded for a period of 24 hours, beginning at the onset of the light phase.

Amplifier and data conditioning

Micro-amplifier

FIG. 30: Pictures of sleep recording room and detail of rotating swivel.

Data analysis. Polygraphic recordings were visually scored by 10 sec epochs. Those epochs were classified as being either wake (W), slow-wave sleep (SWS), or paradoxical sleep (REM), as described earlier (Dugovic et al., 1989). Briefly, the different vigilance states were characterized as follows: WAKE, low-voltage fast EEG activity, high EMG activity; SWS as the block of SWS1 (high-voltage slow cortical waves interrupted by low-voltage fast waves, and reduced EMG activity) and of SWS2 (continuous high-amplitude slow-wave activity in EEG, very low EMG activity); and REM, low-voltage fast cortical waves with a regular theta rhythm, absence of muscular tone. Scores were entered into a computer that calculated various sleep–wake parameters: amount of time spent in the three vigilance states and number and duration of episodes for each state. Sleep–wake

parameters were analyzed over the total light and dark phases. The duration of time spent in the different states of vigilance was expressed in minutes. Parameters of the sleep–wake cycle of control animals were compared with those of the PRS animals under baseline conditions.
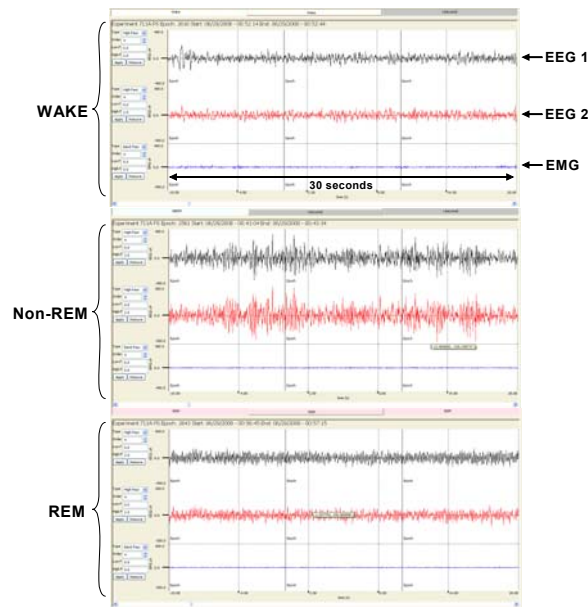
FIG. **31:** EEG and EMG representative of three phases of rat sleep.

7.5 Circadian hypothalamic CRH expression

Hypothalamic incubation

Hypothalamic explants were performed as previously described from Navarra and coworkers (1991). The hypothalami (three per group, six per time point) were then incubated in a 24-well plate (one hypothalamus per well), at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% O_2, in 300 _l MEM with Earle's salts and stable glutamine (Biochrom AG, Berlin, Germany), supplemented with 0.2%

BSA, 0,005% ascorbic acid, and 20 IU/ml aprotinin (pH 7.4). In these experimental conditions, hypothalamic tissues remained viable and functional, as assessed at the end of experiments by measurement of the lactic dehydrogenase activity taken as a marker of cytotoxicity. Thus, variations in CRH release did not appear to be related to non specific tissue damage. After a 60-min preincubation period (during which the medium was changed every 20 min), the medium was replaced with fresh medium alone (control), or medium containing test substances at appropriate concentrations. At the end of the experiments, hypothalami were weighted and the incubation media collected and stored at -35 °C until the measurement of CRH immunoreactivity. To measure intrahypothalamic CRH, the hypothalami were snap frozen and kept at -80 °C, and then homogenized in 1 ml Tris-HCl 50mm (pH 7.4), supplemented with 0.2% BSA and 40 IU/ml aprotinin, using a Teflon glass homogenizer (DuPont Co., Wilmington, DE). For RNA analysis, hypothalami were stored in 2 ml RNA Later TM solution (Ambion, Austin, TX) at -20 °C until RNA extraction.

CRH radioimmunoassay (RIA)

CRH was measured by RIA as previously described from Tringali and coworkers (2006). The detection limit of the assay was 1 pg/tube (100-µl sample volume for incubation media), with intraassay and interassay coefficients of variation of 5% and 10%, respectively. The amounts of both intrahypothalamic and released CRH were expressed as pg/mg wet tissue.

RNA extraction

Total RNA was extracted by the guanidine thiocyanate lysis method of Chomczynski and Sacchi (1987). The average yield of RNA was 45–55 µg/hypothalamus.

RNase protection assay

CRH mRNA expression was measured by the RNase protection assay as previously described in detail (Tringali et al., 2006).

7.6 Assessment of hippocampal neurogenesis

Administration of BrdU

Four month-old animals (twelve per group, only males) were injected 1 time for three days with the thymidine analog bromodeoxyuridine (BrdU, 75 mg/kg/2 ml, i.p.) to label dividing cells. Animals were killed four weeks after the last BrdU injection, four weeks is a sufficient time to permit the study of cells survive. The BrdU is a synthetic thymidine analog that gets incorporated into a cell's DNA when the cell is dividing (during the S-phase of the cell cycle), thus, to study the cells survive several weeks before to kill the animals are necessary, instead few time from the last BrdU injection at the animal death is enough to study the cells proliferation.

Procedure of perfusion and brain cutting

Rats were anesthetized with sodium pentobarbital (60 mg/kg) and perfused with 200 ml saline (NaCl, 0.9%, w/v), followed by 400 ml of cold phosphate buffer (PB, 0.1 M, pH 7.4) containing 4% paraformaldehyde. Samples from all groups were processed in parallel to avoid any non-specific effect of the staining procedure.

Twenty-four hours after postfixation in paraformaldehyde, brains were transferred to 0.1 PB solution and stored until processed for immunohistochemistry. Serial brain sections (40μm) were cut through the whole hippocampus (from -1.81 to -6.1 behind to bregma) (Paxinos e Watson, 1997) using a vibratome (Leica, France) and collected in 0.1 PB.
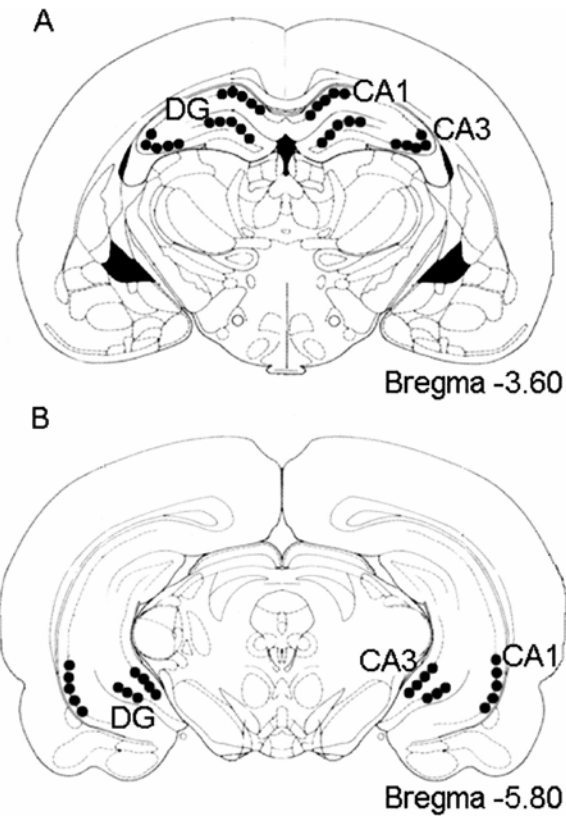
FIG. 32: Illustration of dorsal (A) and ventral (B) hippocampal slides of rat.

Immunohistochemistry

Free-floating sections were processed in a standard immunohistochemical procedure. DNA denaturation was conducted by incubation for 20 min in 2N HCl at 37°C followed by two rinses in 0.1 M borate buffer (pH 8.5). Following several rinses in 0.1 M PB, sections were incubated for 45 min in 3% normal donkey serum (NDS)/ 0.1% Triton X-100 in 0.1 M PB and then incubated for 48h at 4°C with antimouse BrdU (1:500, Roche) diluted in 1% NDS/0.1 M PB. Sections were then incubated for 2 hours with secondary antibody (biotinylated-donkey-antimouse 1:500, Jackson Immuno Research) diluted in PBS-Tds. After washing in 0.1 M PB, sections were incubated for 1h in avidin-biotin-peroxidase complex (ABC Elite kit, Vectastain) and rinsed in 0.1 M PB. Peroxidase was detected using glucose oxidase-DAB-nickel method (Shu et al, 1988). Sections were rinsed in water, then washed

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repeatedly in 0.1 M PB, mounted onto gelatin coated slides, air-dried for 30 min, dehydrated in ethanol, cleared in xylene and covershipped using Eukitt (O Kindler, Freiburg, Germany).

Quantification

All analyses were performed blindly using coded sections. Every sixth in a series of 40-μm sections was analyzed: an average of 18 sections was analyzed per animal. All BrdU-immunoreactive cells were bilaterally counted under the microscope with a 40x and 80x objective in the subgranular zone (SZG) and in the granule cell layer (GCL) of the dentate gyrus. The quantification was conducted over the two hemi-hippocampi and expressed as the summed-up number of BrdU labeled cells over SGZ and GCL. The number of BrdU labeled cells was examined separately in the dorsal (-3.12 to 4.20 mm from bregma; Paxinos and Watson, 1986) and ventral (-4.36 to -6.3 mm to bregma) hippocampus. Means for dorsal and ventral regions were obtained from 9 sections per rat, respectively.

7.7 Western blot analysis

Eight animals per group (controls and PRS, males) of different age (PND 6, PND 10, PND 22, PND 32, PND 42, PND 100, 10 months old) were used and analyses performed in duplicate. Rats were killed by decapitation and brains rapidly removed; hippocampi were dissected and stored at -80°C. On the day of the experiment, tissue was homogenized at 4°C with a polytron in 500 ml of lysis buffer (100 mM Tris buffer, phenylmethylsulfonyl fluoride 1 mM, leupeptin10 mg/ml and aprotinin 10 mg/mL pH 7.2). Protein concentrations were determined using the Bradford protein assay (Bradford, 1976). Forty micrograms of protein were resuspended in sodium dodecyl sulphate (SDS)-bromophenol blue reducing buffer with 40 mM dithiothreitol (DTT).

The samples were separated on 8% SDS-polyacrylamide gels (Amersham Bioscience, Inc., Little Chalfont, England) and after electrophoresis (Mini-PROTEAN 3 System, Bio-Rad, Hercules, CA, USA), the proteins were transferred to nitrocellulose membranes (Amersham Bioscience) using 35 mM Tris, 192 mM glycine and 20% methanol for 4 h. Samples from control and PRS rats migrated on the same gel. After transfer, blots were incubated in a solution (blocking solution) containing Tris-buffered saline (TBS), 0.5% (w/v) Tween-20, 1% (w/v) non-fat milk and 1% (w/v) bovine serum albumin. Subsequently, blots were incubated overnight with rabbit anti-mGluR1 (1:500), rabbit anti-mGluR5 (1:1000), mGluR2/3 (1:1000) (Upstate Biotechnology, Lake Placid, NY, USA), in blocking solution at 4°C.. After incubation with the primary antibody, blots were washed three times with TTBS buffer. Then, the filters were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:10.000; Amersham Bioscience) for 1h at room temperature (21° C ±2). To ensure that each lane was loaded with an equivalent amount of protein, the blots were probed with an anti-actin serum (1:1000; Sigma, St Louis, MO, USA) overnight at 4° C. Subsequently, blots were incubated with horseradish peroxidase- conjugated goat anti-mouse antibodies (1:5000; Amersham Bioscience) for 1h at room temperature. Immunoreactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences).

After immunoblotting, digitized images of bands immunoreactive for target (mGluR1 or mGluR5 or mGluR 2/3) and control (actin) molecules were acquired and the area of immunoreactivity corresponding to each band was measured using the Scion Image computer program (Scion Corp., Frederick, MD, USA). A ratio of target to actin was then determined, and these values were compared for statistical significance.
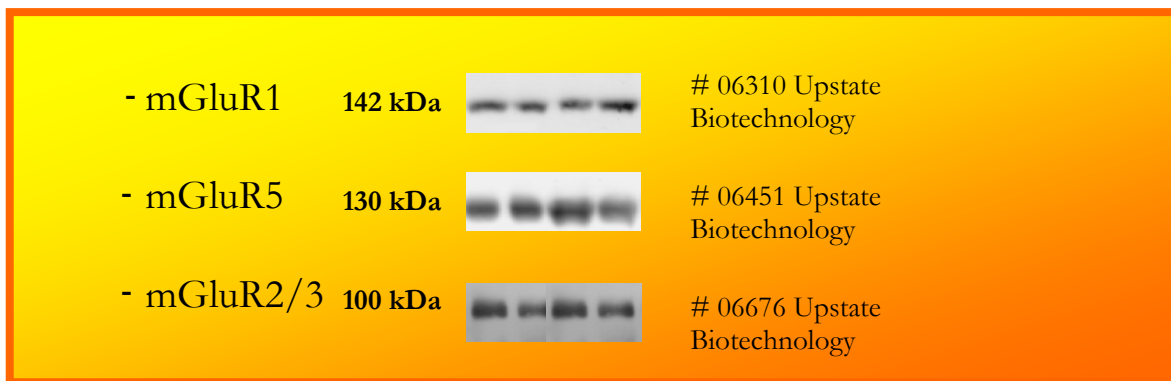
FIG. 33: Schematic view of mGlu receptors analyzed in adult hippocampus of rat

7.8 Hippocampal Proteome

Sample preparation for 2D electrophoresis

PRS and control male rats at 8 months of age were killed and their brains removed. Hippocampi were rapidly dissected, frozen on dry ice and stored at -80°C.

Then, hippocampal samples were 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), 3 mM tributylphosphine (Fluka), 2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]- 1-propanesulfonate, CHAPS (Fluka), 1% Pharmalytes 3.5–10 (Amersham Biosciences, Uppsala, Sweden), Complete ™ protease inhibitor (Roche, Basel, Switzerland). Samples were sonicated three times for 10 seconds 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. Proteins in the supernatant were separated by 2-D electrophoresis. An aliquot of this supernatant was used to measure protein concentration by the Bradford Method.

2-D electrophoresis: Isoelectric focusing was carried out on 17 cm immobilized pH gradient strips (3–10 non-linear pH gradient, Bio-Rad, Hercules, CA, USA). The strips were rehydrated for 16 h with 0.4 mg protein in 450 _L of the solubilizing

solution already described, with the addition of 10 mM iodoacetamide (Sigma-Aldrich) as alkylating agent (Herbert et al., 2001). Focusing was carried out at 20 °C for 75,000 Vh at a maximum of 10,000 V in a Protean IEF Cell (Bio-Rad). Immobilized pH gradient strips were then incubated with gentle shaking in an equilibration solution (6 M urea, 2% sodium dodecyl sulfate, 375 mM Tris pH 8.8, 4 mM tributylphosphine) for 20 min. The strips were then laid on top of homemade 10% polyacrylamide gels (acrylamide from Bio-Rad) in the presence of 0.1% agarose (Sigma- Aldrich) prepared in running buffer and stained with Bromophenol Blue (Sigma-Aldrich). An aliquot of Precision™ mass markers (Bio-Rad) was loaded in parallel on each gel. Gels were run (Genomics Solutions) at 18 °C and 12 mA/gel. After overnight fixation staining was carried out with silver stain (Bio-Rad). Gels were destained 1 h in a 10% v/v ethanol and 7% (v/v) acetic acid solution and maintained in water. Images were acquired with a Biorad densitometer system. Image analysis was carried out with the Progenesis software (Bio-Rad). Protein levels were evaluated as volumes (spot area_optical density) for the protein spots matched among gels. Spot volume was normalized for each gel on total density in valid spots. Data were log transformed and analyzed with Student's *t*-test with the statistics tools included in the Progenesis software. Spots which gave significant results (*P*_0.05) were verified visually to exclude artefacts.

Protein identification with LC-MSMS Each selected spot was carefully cut with a spot cutter (Bio-Rad) and destained with two 10 min-washing steps in 50% Acetonitrile (Sigma-Aldrich) (v/v), 50% of 5 mM Tris, pH 8.5, followed by a third wash with 5 mM Tris pH 8.5 for 10 min. The spots were dried in a Speedvac sc110A device (Thermo Savant, NY, USA) for 1 h at room temperature and then covered with 15 _L of sequencing grade modified trypsin (Sigma-Aldrich) (0.02 mg/mL) in NH4HCO3 buffer (40 mM, pH 8.5) and left at 37 °C overnight. The spots were then crushed and peptides were extracted twice in 50 _L 50%

acetonitrile, 50% H2O with 1% formic acid (v/v) and a third time with 50 _L acetonitrile. The extractions were conducted in an ultrasonic bath for 15 min. The three extraction solutions were mixed and evaporated to dryness in the Speedvac device and the residues dissolved in 10 _L H2O with 0.1% trifluoroacetic acid. For an additional purification, the samples were cleaned by using ZIP-TIP C18 (Millipore, Bedford, MA, USA). Two microliters of the resulting solution were mixed with an equivalent volume of matrix solution, prepared fresh every day by dissolving 10 mg/mL _-cyano- 4-hydroxycinnamic acid (Sigma-Aldrich) in acetonitrile:ethanol (1:1, v/v). One microliter of the resulting mixture was loaded onto the MALDI sample plate and allowed to dry. Measured peptide masses were analyzed by two different software tools, which allowed a double confirmation of protein identification: MASCOT software (Matrix Science London, UK, MS/MS ions search module) which incorporates a probability-based scoring, was used to identify proteins, using Swiss-Prot database with Rattus as taxonomic category. The search parameters were 1.5-Da tolerance for the parent ion mass and 0.8 Da for the MS/MS fragment ions, one missed cleavage allowed, carbamidomethylcysteine as fixed modification, and methionine oxidation as possible modification. Only the peptides with a significant Mascot score (>28) were considered and reported after manual verification of the fragmentation spectra.
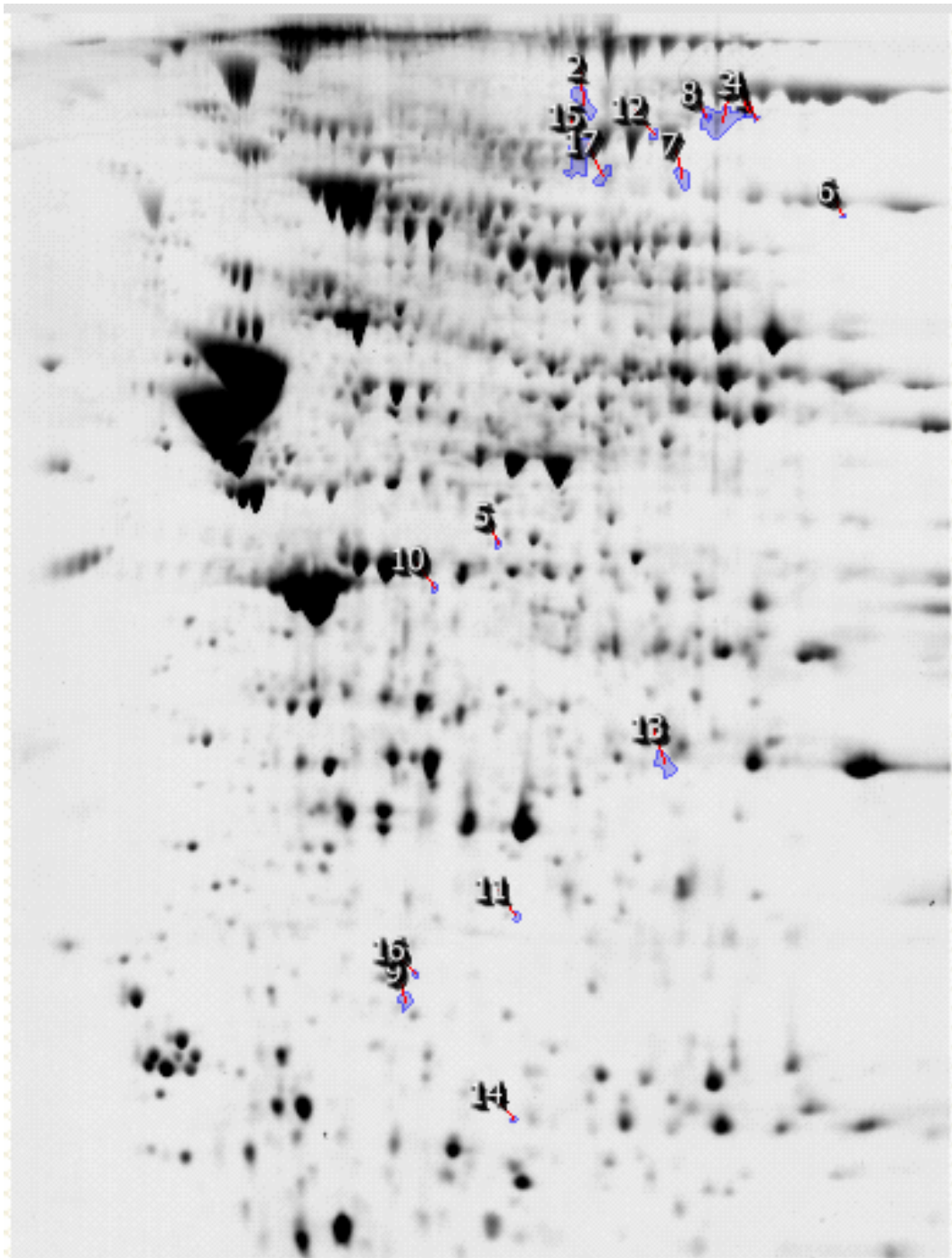
FIG. 34: 2DE gel of rat hippocampus proteins. The spots analyzed by LC-MS/MS are arrowed.

7.9 Chronic treatment with AG0310 on locomotor activity

Antidepressant treatment

The animals (12 per group) were 4 months old when the experiment started and about 7 months when it finished. The AG0310, a new antidepressant in testing was added to powder food (2000 ppm) at dose of 40mg/kg. The treatment lasted five weeks and the animals of control group received the same powder food, but, clearly, without antidepressant. For all treatment duration, the animal's weight was observed.

Experimental design

The exact experimental design is depicted in FIG. 35. Before the five weeks of AG0310 treatment, animals were habituated for five weeks at powder food and at the cages equipped with a running wheel. After the habituation there were ten consecutive days of locomotor activity analysis. Then, the animals were treated for five weeks with AG0310 or placebo, and therefore, again 10 consecutive days of analysis. All data collected were scores as previous described.
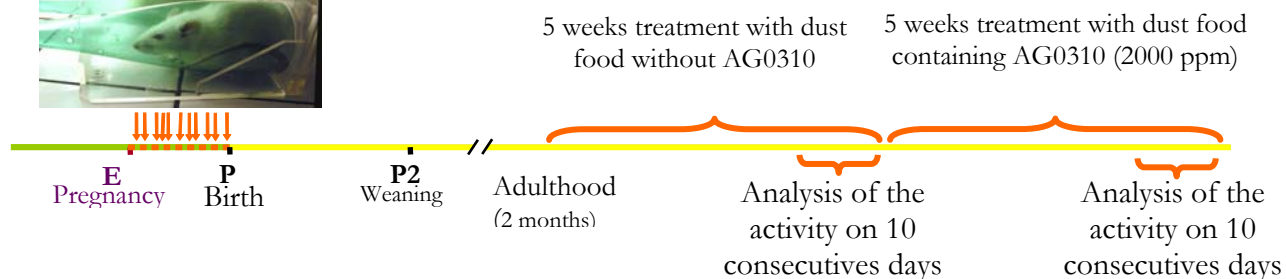
FIG. 35: A schematic view to explain the experimental protocol used to study the effect of chronic AG0310 administration to reverse the PRS alterations on locomotor activity.

7.10 Chronic treatment with AG0310 on sleep/wake cycle

Antidepressant treatment

The rats (10 per group) were 2.5 months at the time of the experiment. The electrode implantation procedure was previous discussed. After that polysomnographic recording of PRS and control rats was performed 24 consecutive hours in basal condition (two weeks after the surgery and without any treatment), there was three weeks of AG0310 or vehicle treatment. The AG0310 was added to powder food (2000 ppm, 40mg/kg), and the animals were weighted for all treatment time.

Experimental design

After electrode implantation there were two weeks to permit post-operative recovery and the habituation to powder food and to the recording cylinders. Then, for 24 consecutive hours a sleep/wake cycle was recorded to have a datum point in basal condition. Three weeks of chronic oral treatment with AG0310 or vehicle follow, and again, a polysomnographic recording of all animals for 24 consecutive hours was achieved. The data was, therefore, scored as previous described. The exact experimental design is depicted in the following FIG. 36.

3 weeks of chronic oral treatment with AG0310

Week 1   Week 2   Week 4   Week 5

Post-operative recovery

Habituation to the cylinder and powder food

Sessions of habituation to connection of the **system** of recording

24 hours continuous recording for the analysis
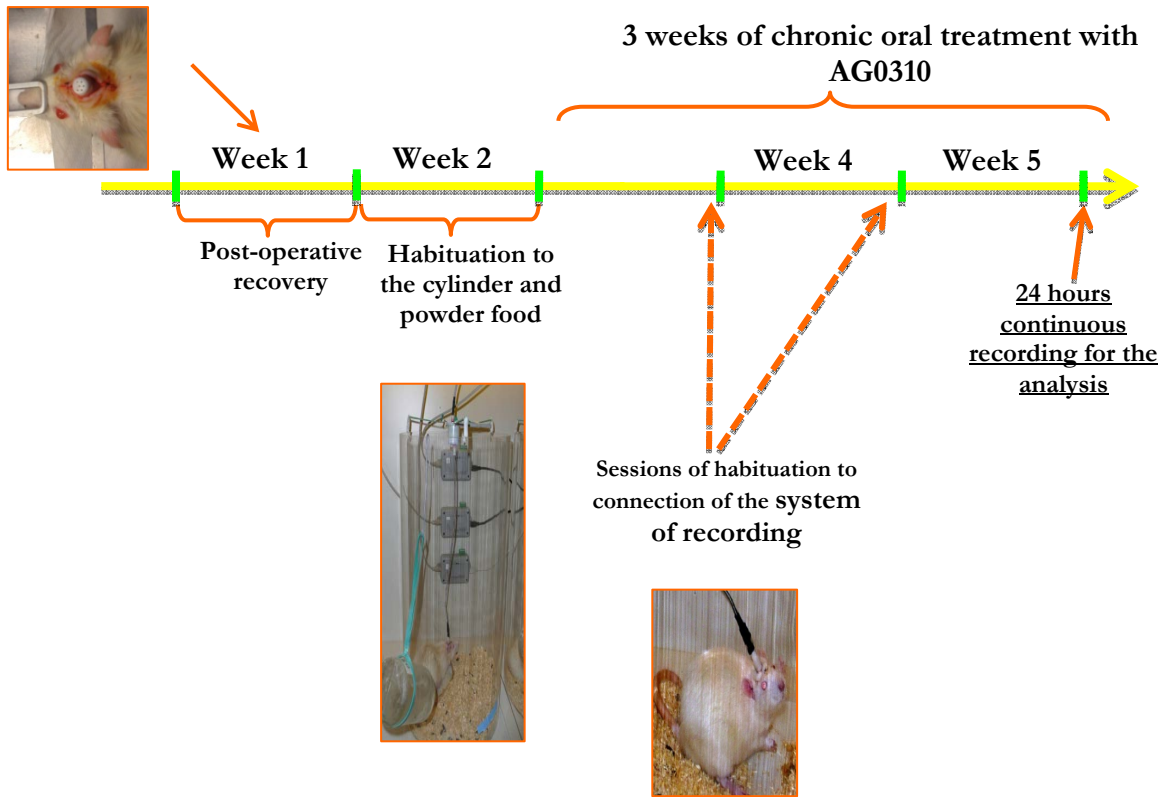
FIG36: A schematic view of the experimental protocol to evaluate the effect of chronic treatment with AG0310 to contain the PRS alteration on sleep/wake cycle.

7.11 Chronic treatment with fluoxetine treatment on hippocampal neurogenesis

Antidepressant drug administration

Animals (12 per group) were aged 4 months when treatment started and about 5 month old when sacrificed for immunohistochemical analysis. Vehicle rats received injections of saline solution (NaCl, 0,9%), in a volume of 2ml/kg. Fluoxetine (Sigma, France) was dissolved in saline solution and intraperitoneally injected (2ml/kg) at the dose of 5mg/kg. This dose has been chosen based on antidepressant properties of the drug and on previous results on neurogenesis in basal conditions (Malberg et al., 2000). Drug and vehicle were administered, once daily, for 4 weeks. Injections were performed at 16h00.

Experimental design

The exact experimental design is depicted in FIG: 37. Before the four weeks of fluoxetine administration, animals were injected 1 time a day for three consecutive days with the thymidine-analog-bromodeoxyuridine (BrdU, 75 mg/kg/3ml i.p.) to label dividing cells. At the end of treatment, 24 hours after the last antidepressant injection, animals were terminally anaesthetized with sodium pentobarbital (60 mg/kg i.p.) and then perfused. Samples from each treatment group were processed for the immunohistochemistry (the procedure was previous described).
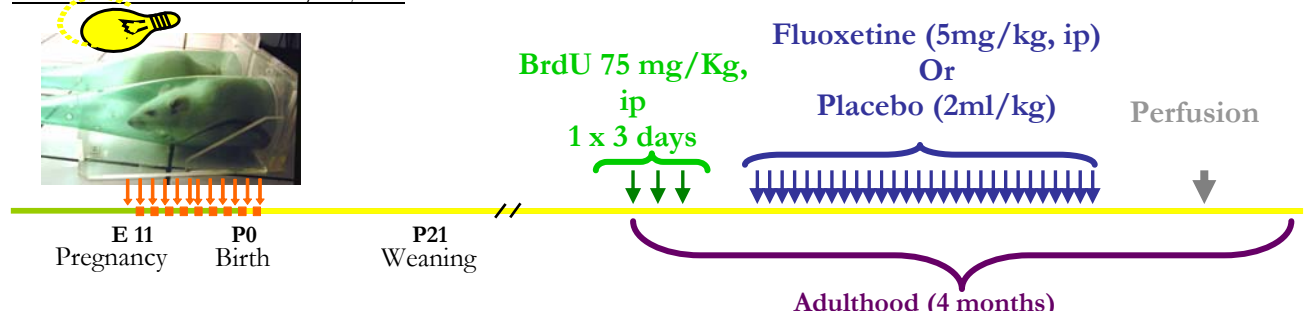
FIG:37: A schematic view of experimental protocol used to study the effect of chronic fluoxetine treatment on the reduced neurogenesis provoked by PRS.

7.12 Statistical analysis

Except for BrdU quantification, before and after antidepressant treatment, all other examined parameters were analyzed with the Student's T-Test. The probability value (p) $\leq$ 0.05 was considered to indicate a significant difference between the considered groups. Data concerning hippocampal neurogenesis, expressed as mean value $\pm$ SEM, were analyzed using parametric analysis of variance (ANOVA), with group (Control *vs* Prenatal Stress) and treatment (Vehicle *vs* Fluoxetine) as between-subject variables, followed by Newman-Keul's post-hoc comparisons for further examination of group's differences. Similar analyses (three ways ANOVA) were applied when sub-regional analyses were performed by adding Region as independent variable. The level of significance was set at $p<0.05$.

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