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by

Marie-Line REYNAERT

STRESS, SEX AND ADDICTION:

SELF-MEDICATION HYPOTHESIS IN THE RESPONSE TO REWARD IN THE PRENATAL RESTRAINT STRESS MODEL IN RAT

Members of the Jury:

Dr. François TRONCHE	Univ. Pierre and Marie Curie, Paris, France	***President of the Jury***
Dr. Serge AHMED	Institute of neurodegenerative diseases, Bordeaux 2, France	***Examiner***
Prof. Tracy BALE	Univ. of Pennsylvania, Philadelphia, USA	***Examiner***
Prof. Mohamed KABBAJ	Florida State Univ., Tallahassee, USA	***Reporter***
Prof. Ron De KLÖET	Dpt of Medical Pharmacology, Leiden Univ., The Netherlands	***Reporter***
Prof. Rainer LANDGRAF	Max Plank Institute of Psychiatry, Munich, Germany	***Reporter***
Prof. Ferdinando NICOLETTI	La Sapienza Univ. of Rome, Italy	
	Univ. Lille 1, France	***Internship reporter***
Prof. Stefania MACCARI	Univ. Lille 1, France	***Thesis supervisor***

Invited member:

Dr. Sara MORLEY-FLETCHER	Univ. Lille 1, France	***Thesis supervisor***

PRENATAL STRESS
AND NEURODEGENERATIVE DISEASES
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ABSTRACT

Stress is an important factor in the etiology of mood disorders and addictive behaviors. Prenatally restraint stressed (PRS) rats, i.e. the offspring of dams submitted to repeated episodes of stress during the last ten days of gestation display stress-related disorders such as anxiety- and depressive-like disorders but also increased vulnerability to drugs of abuse. An impairment of glutamate release in the ventral hippocampus lies at the core of the anxiety-like profile of PRS rats. Hence, we decided to investigate the effect of antidepressant treatment on glutamatergic system in our model. We found that chronic treatment with classical antidepressant drugs was able to enhance glutamate release and correct anxious-/depressive like profile of PRS male rats. Of note, a clear-cut sex effect has been shown in PRS-induced profile, with males being more anxious while male and female PRS rats display a similar depressive-like behavior. Here, for the first time, the alteration of circadian patterns, as a feature of depressive-like phenotype was analyzed both in male and female rats, and we have shown a gender-specific outcome of PRS on circadian systems modulating locomotor activity, the resynchronization to the new light-dark cycle, and hypothalamic CRH levels. Also concerning addiction, female profiles are less studied, so, we extended the impact of sex in our model to addiction. We have demonstrated that sex and in particular sex hormones played a key role in determining rats preference for drugs in a conditioned place preference paradigm, and that sensitiveness was expressed in a stimulus-dependent manner (chocolate as natural reward in comparison to cocaine). Finally, we found an enhanced preference for cocaine in females and in PRS rats of both sexes. This increase in preference for cocaine was linked to locomotor activating effect but also to the anxiolytic and antidepressant effect of the drug. This suggests that preference for a drug is enhanced when animals found a beneficial effect of this drug in correcting their mood disorders, reinforcing the hypothesis of self-medication in addictive-like disorders.

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STRESS, SEXE ET ADDICTION:

HYPOTHESE DE L'AUTO-MEDICATION DANS LA REPONSE AU RENFORCEMENT DANS LE MODELE DE STRESS PRENATAL CHEZ LE RAT

Membres du Jury:

Dr. François TRONCHE	Univ. Pierre et Marie Curie, Paris, France	***Président du Jury***
Dr. Serge AHMED	Institut des troubles neurodégénératifs, Bordeaux 2, France	***Examineur***
Pr. Tracy BALE	Univ. de Pennsylvanie, Philadelphie, USA	***Examineur***
Pr. Mohamed KABBAJ	Univ. De Floride, Tallahassee, USA	***Rapporteur***
Pr. Ron De KLÖET	Dpt de Pharmacologie Médicale, Univ. de Leiden, Paus-Bas	***Rapporteur***
Pr. Rainer LANDGRAF	Max Plank Institut de Psychiatrie, Munich, Allemagne	***Rapporteur***
Pr. Ferdinando NICOLETTI	La Sapienza Univ. de Rome, Italie	
	Univ. Lille 1, France	***Rapporteurde stage***
Pr. Stefania MACCARI	Univ. Lille 1, France	***Directrice de Thèse***

Member Invité:

Dr. Sara MORLEY-FLETCHER	Univ. Lille 1, France	***Directrice de Thèse***

Université Lille 1 Sciences et Technologies

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RESUME

Le stress est un facteur d'importance dans l'étiologie des troubles de l'humeur et des comportements addictifs. Des rats stressés prénatalement (PRS: prenatal restraint stress), i.e. la progéniture de femelles soumises à des épisodes répétés de stress les dix derniers jours de gestation, présentent des troubles liés au stress telles que des affections de type anxiété/dépression mais également une vulnérabilité aux drogues. Une diminution de la libération de glutamate dans l'hippocampe ventral s'avère au cœur du profil d'anxiété des rats PRS. Aussi, nous avons entrepris d'étudier dans notre modèle l'effet d'un traitement antidépresseur (ATD) sur le système glutamatergique. Nous avons montré qu'un traitement chronique avec des ATDs classiques était en mesure d'améliorer la libération de glutamate et de corriger le profil anxiodépressif des rats PRS mâles. Notons qu'un effet net du sexe a été mis en évidence dans le profil induit par le PRS, les mâles étant plus anxieux alors que les rats PRS mâles et femelles présentent un comportement de type dépressif similaire. Ici, pour la première fois, la modification des patterns circadiens, comme caractéristique de la dépression, a été analysée dans une même étude à la fois chez les rats mâles et femelles. Ainsi, nous avons montré un effet spécifique du sexe concernant l'impact du stress prénatal sur les systèmes circadiens, modulant l'activité locomotrice, la resynchronisation pour un nouveau cycle lumière-obscurité, et les niveaux de CRH hypothalamique. Nous avons ensuite étendu l'étude de l'influence du sexe dans notre modèle à la dépendance. Nous avons établi que le sexe, et en particulier les hormones sexuelles, jouent un rôle clé dans la détermination de la préférence des rats pour les drogues dans un paradigme de préférence de place conditionnée, et que la sensibilité était exprimée d'une manière stimulus-dépendante (chocolat comme récompense naturelle, comparativement à la cocaïne). Enfin, nous avons constaté que l'augmentation de la préférence pour la cocaïne était liée à l'effet d'activation de la locomotion, mais aussi à l'effet anxiolytique et antidépresseur de la drogue. Ceci suggère que la préférence pour une drogue est augmentée lorsque les animaux ressentent un effet bénéfique de cette drogue dans l'amélioration de leurs troubles de l'humeur, renforçant l'hypothèse de l'automédication dans les problèmes d'addiction.

Neural plasticity Team, Head Prof. Stefania Maccari,
UMR8576/CNRS- Unit of Glycobiology Structural and functional (UGSF),
Head Prof. Christophe D'Hulst

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Directors: Prof. Stefania Maccari and Prof. Ferdinando Nicoletti

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List of publications

The work of my thesis has been summarized in the following publications:

- Article 1- J Marrocco, **ML Reynaert**, E Gatta, C Gabriel, E Mocaer, S Di Prisco, E Merega, A Pittaluga, F Nicoletti, S Maccari, S Morley-Fletcher and J Mairesse. The effects of antidepressant treatment in prenatally stressed rats support the glutamatergic hypothesis of stress-related disorders. J Neurosci 2014, 34: 2015-2024.

- Article 2- G Van Camp, **ML Reynaert**, E Gatta, C Laloux, A Tramutola, C Cinque, P Navarra, S Morley-Fletcher, F Nicoletti, S Maccari and J Mairesse. Altered circadian locomotor activity in male and female rats in the prenatal restraint stress model of depression, *In revision*

- **ML Reynaert**, G Van Camp, A Mullier, J Marrocco, H Bouwalerh, J Mairesse, S Maccari, F Nicoletti, S Morley-Fletcher. Role for sex hormones in prenatal stress-induced programming of preference to natural reward. Society for Neuroscience, 2012.

- Article 3- **ML Reynaert**, J Marrocco, L Lionetto, L Deruyter, D Allorge, A Moles, S Maccari, S Morley-Fletcher, G Van Camp and F Nicoletti. Sex hormones mediate the effect of early life stress on natural reward, *submitted*

- Article 4- **ML Reynaert**, E Gatta, J Marrocco, G Van Camp, J Mairesse, F Nicoletti, S Maccari, S Morley-Fletcher. Locomotor activity response to cocaine is predictive for drug-induced conditioned place preference: sex and stress as modulating variables, *In preparation*

- Article 5- **ML Reynaert**, E Gatta, J Marrocco, J Mairesse, G Van Camp, F Nicoletti, S Morley-Fletcher, S Maccari. Antidepressant-like effect of cocaine is associated with increased reward in male and female prenatally stressed rats, *In preparation*

- Book chapter- **ML Reynaert**, J Marrocco, E Gatta, J Mairesse, G Van Camp, F Fagioli, S Maccari, F Nicoletti and S Morley-Fletcher. A self-medication hypothesis for increased vulnerability to drug abuse in prenatally restraint stressed rats. "Perinatal Programming of Neurodevelopment", Book chapter 6, Editor: MC Antonelli – Springer, *In edition.*

During my PhD program, I was also involved in the following studies

- J Mairesse, G Van Camp, E Gatta, J Marrocco, **ML Reynaert**, M Consolazione, S Morley-Fletcher, F Nicoletti and S Maccari. Sleep in prenatally restraint stressed rats, a model of anxious depression. "Perinatal programming of Neurodevelopment", Book chapter 2, Editor: MC Antonelli – Springer, *In edition.*

- J Mairesse, E Gatta, **ML Reynaert**, J Marrocco, S Morley-Fletcher, M Soichot, L Deruyter, G Van Camp, H Bouwalerh, F Fagioli, A Pittaluga, D Allorge, F Nicoletti and S Maccari.Activation of oxytocin receptors restrains both the development and expression of the pathological programming induced by prenatal stress in rats, *In revision*

STRESS, SEX AND ADDICTION:

SELF-MEDICATION HYPOTHESIS IN THE RESPONSE TO REWARD IN PRENATAL RESTRAINT STRESS MODEL IN RAT

GENERAL SUMMARY

Addiction, or compulsive drug seeking, has become a very important health concern, and about 200 million adults worldwide would use illicit drugs, including cannabis, amphetamines, cocaine, and opioids such as heroin. In addition, there is mounting evidence for an epidemy of behavioral addictions in addition to substance abuse. This concerns compulsive behaviors towards natural stimuli, such as food, which are referred to as nondrug addiction. It was demonstrated that the sensibility to food can surpass the response to psychostimulant drugs leading some individuals to describe their addiction to food. Nevertheless, this account remains controversial.

Of note, people are not equal regarding drug addiction: genetic inheritance, as well as epigenetic factors, intervene in humans propensity to drug seeking, with some individuals displaying a vulnerable profile. Interestingly, in animal models, the same phenomenon is described with two distinct phenotypes revealed: some animals show a high sensitiveness toward drugs of abuse whereas others are resistant. Events occurring during the early life can highly program neurobehavioral alterations all along the life span. Traumatic experiences endured in childhood (war, abuses, violence) are for instance an important risk factor for the occurrence of addictive-like behaviors. Interestingly, sex differences exist in the vulnerability to addiction. Women are more sensitive to drugs effects and, when they have a first experience of drug abuse and avowed addictive behavior, the risk of relapse is high after a withdrawal period, although women consume less. Of note, in general, a high prevalence of mood disorders is found in women in comparison to men. And, the enhanced propensity to relapse would be associated with an exaggerated response to withdrawal symptoms in women with exacerbation of feelings of stress and depression. In female rats, the same enhanced sensitiveness to drugs is established. One explanation would be the intervention of sex hormones, namely estrogens, as, after ovariectomy, sensitiveness to drugs of abuse is decreased and estradiol replacement therapy is able to reinstate the vulnerability. In males, the question of sex hormones in drug addiction has also been addressed, but the results are still quite conflicting and the real impact of testosterone is not yet established; some authors report that orchidectomy is able to reduce sensitiveness to reward whereas others demonstrate no effect.

In humans, a high comorbidity between anxiety/depression and addiction is reported. In patients suffering from drug abuse, a high prevalence of anxious-depressive symptoms is found and antidepressant therapy appears efficient in treating addictive behavior. This clinical

evidence supports the “self-medication” hypothesis that suggests drug seeking behavior as a secondary symptom of depression.

We have shown that the Prenatal Restraint Stress (PRS) model in rats is a model that recapitulates features of stress-related disorders, with high construct, face and predictive validity. Thus, PRS rats, i.e. the offspring of rats submitted to restraint stress during the last ten days of gestation, display dysfunctions in the hypothalamic-pituitary-adrenal (HPA) axis anxious-/depressive-like phenotype, alterations of sleep-wake cycle. Alterations in he hippocampal glutamatergic system, glutamate being the major excitatory brain neurotransmitter, was found to lie at the core of the anxious-/depressive-like profile of PRS rats. The PRS-induced behavioral alterations were found to be corrected with antidepressant treatment. An enhanced sensitiveness to the psychostimulant effect of drugs of abuse, with increase vulnerability to locomotion activating effect as well as propensity to self-administration has also been described in PRS rats. Interestingly, the neurobehavioral profile of PRS rats, associated to anxiety was found to be sex-dependent, with males being more anxious than females while depressive-like phenotype appears similar in male and female PRS rats. But, until now, little is known concerning a putative sex-dependent effect in drug addiction in the PRS model.

The objective of my thesis was to bring further elements in the **characterization of both anxious-/depressive-like behavior and addictive-like disorders in the PRS rat model**. I also attempted to evaluate the **role of sex and in particular sex hormones in modulating phenotypes** in male and female control unstressed and PRS rats. Then, I examined how the greater sensitiveness to drugs of abuse observed in PRS rats could be explained by an **anxiolytic/antidepressive effect of drugs of abuse**, in a **self-medication strategy**.

In chapter one, we have brought further arguments in favor of the glutamatergic hypothesis of anxious-/depressive-like phenotype of PRS rats. Indeed, we have shown that the efficacy of antidepressant drugs in correcting PRS-induced behavioral abnormalities passed through a correction of the glutamatergic system (**article 1**). We have also studied circadian patterns of locomotor activity, with disturbances in circadian rhythms as a feature of depressive-like phenotype, and we report that both male and female PRS rats displayed depressive-like symptoms (**article 2**). We also demonstrate that sex hormones play a key role in anxiety and depression.

In chapter 2, we extended the question of sex and sex hormones to drug addiction. We first examined whether PRS would influence sensitiveness to both psychostimulant drug (cocaine) (**article 4**) and natural reward chocolate (**article 3**). This last point was important to demonstrate that a natural stimulus could be a drug. In this part, we have used a conditioned place preference paradigm (CPP), which consist in pairing a drug to a particular environmental context (contextual cue). We have shown that cocaine CPP was enhanced in PRS animals of both sexes while PRS enhanced chocolate CPP in male and not female rats.
Sex hormones modulated reward-induced CPP. Behavioral data found neuronal correlates; in particular serotonin in hypothalamus and dopamine in the nucleus accumbens and prefrontal cortex were affected by PRS, sex and sex hormones, in a way that fitted nicely with preference for chocolate (article 3).

Another issue of this work was to better establish a possible link between stress-related disorders, and propensity to drug use. PRS model, which meets criterion of both anxio-depressive and addictive-like disorders, represents a very potent model to address this question.

So, **in chapter 3**, we have studied the effect of chronic cocaine administration on the anxious-/depressive–like phenotype of Control and PRS male and female rats. We report that cocaine was able to correct anxious-/depressive-like symptoms, in a disease-dependent manner. Glutamate appears a key regulator of the effects underlined.

Taken together, our results indicate that PRS model is a well integrated model of multiple inter-related pathologies, in which sensitiveness to drugs of abuse would be a self-medication strategy to alleviate symptoms of anxiety/depression, and in which gender and in particular sex hormones are key actors of the mediation of all these comorbid stress-related disorders.

RESUME GENERAL

L'addiction, ou la quête compulsive de drogue, est devenue un problème majeur de santé publique, et environ 200 millions d'adultes à travers le monde feraient usage de drogues illicites, telles que le cannabis, les amphétamines, la cocaïne, et les opiacés comme l'héroïne. Le problème de l'addiction apparait d'autant plus important que, parmi les récompenses, si la sensibilité aux substances d'abus est évidemment décrite, il existe également de plus en plus d'arguments en faveur de l'existence de dépendances comportementales. Un comportement compulsif envers des stimuli naturels, tels que les aliments, appelé dépendance sans produit, reçoit ainsi de plus en plus d'attention, avec des expérimentations montrant que la sensibilité aux aliments peut surpasser la réponse à des psychostimulants, et de nombreux témoignages de personnes décrivant leur addiction alimentaire. Toutefois, cette considération reste controversée.

Notons que nous ne sommes pas égaux face à la toxicomanie: l'héritage génétique, ainsi que des facteurs épigénétiques, interviennent dans la propension de l'homme à la recherche de drogue, avec certains individus affichant un profil de vulnérabilité. Fait intéressant, dans des modèles animaux, le même phénomène est décrit avec deux phénotypes distincts révélés: certains animaux montrent une grande sensibilité envers les drogues d'abus alors que d'autres apparaissent résistants. Les événements survenant au cours de la vie précoce peuvent très fortement programmer des altérations neurocomportementales tout au long de la vie. Des expériences traumatiques subies dans l'enfance (guerre, abus, violence) sont par exemple un facteur de risque important pour l'apparition de comportements de dépendance.

Fait intéressant, des différences sexuelles existent dans la vulnérabilité aux drogues: bien que les femmes consomment moins, celles-ci sont plus sensibles aux effets des drogues et, après une première expérience de la toxicomanie et une conduite addictive avérée, le risque de rechute est élevé après une période de sevrage. Cette propension accrue à la rechute serait associée à une réaction exagérée aux symptômes de sevrage chez les femmes avec l'exacerbation des sentiments de stress et de dépression. Chez les rats femelles, le même phénomène est observé. Après une période d'extinction (ou abstinence) suite à une procédure d'auto-administration, une dose plus faible de la drogue est nécessaire pour rétablir la réponse chez les femelles comparativement aux mâles. Aussi, la réponse à la drogue dans un protocole de préférence de place conditionnée, où un stimulus renforçant par rapport à un stimulus neutre sont associés à deux compartiments différents d'un apparat durant les sessions de conditionnement, est augmentée chez les femelles. Une explication serait l'intervention des

hormones sexuelles, à savoir l'estradiol, car les études montrent que, après ovariectomie, la sensibilité aux drogues d'abus est diminuée et la thérapie de remplacement en estradiol est en mesure de rétablir la vulnérabilité. Chez les femmes, un tel impact des hormones sexuelles est également décrit, avec des fluctuations de la sensibilité au désir de la drogue et aux symptômes de sevrage en fonction du cycle menstruel. Chez les mâles, la question des hormones sexuelles dans la toxicomanie a également été abordée, mais les résultats sont encore très contradictoires et l'impact réel de la testostérone n'est pas encore établi; certaines études rapportent que l'orchidectomie est capable de réduire la sensibilité aux drogues tandis que d'autres ne montrent aucun effet.
Chez l'homme, une comorbidité élevée entre anxiété/dépression et toxicomanie est rapportée. Chez les patients souffrant d'abus de drogues, une forte prévalence de symptômes anxio-dépressifs est reportée et un traitement avec des antidépresseurs semble efficace dans le traitement des conduites addictives. Cette évidence clinique confirme l'hypothèse de l'"automédication" qui suggère le comportement de recherche de drogue comme symptôme secondaire de la dépression.

Dans ce contexte, le modèle de stress prénatal (PRS: prenatal restraint stress) chez le rat est un modèle qui récapitule les caractéristiques de troubles liés au stress, avec une grande validité de construction, d'apparence et prédictive. Ainsi, les rats PRS, à savoir la progéniture de rates soumises à un stress de contention au cours des dix derniers jours de gestation, affichent un phénotype de type anxio-dépressif, des modifications du cycle veille-sommeil et une sensibilité accrue à l'effet psychostimulant de drogues, avec une augmentation de la sensibilité à l'effet d'activation de la locomotion et une vulnérabilité pour l'auto-administration. De manière intéressante, le profil neurocomportemental des rats PRS associé à l'anxiété s'est avéré dépendant du sexe, les mâles étant plus anxieux que les femelles alors que le phénotype de type dépressif apparaît similaire chez les deux sexes. Mais, jusqu'à présent, rien n'est connu concernant un possible effet du sexe dans l'addiction aux drogues dans notre modèle de PRS.

L'objectif de ma thèse était d'apporter de nouveaux éléments dans la **caractérisation des comportements à la fois de type anxio-depressif et les troubles addictifs dans le modèle PRS chez le rat**. J'ai aussi évalué le **rôle du sexe et en particulier des hormones sexuelles dans la modulation de phénotypes** chez les rats contrôles non stressés et PRS mâles et femelles.

Ensuite, j'ai examiné dans quelle mesure la plus grande sensibilité aux drogues d'abus observée chez les rats PRS pourrait s'expliquer par un **effet anxiolytique et/ou antidépresseur des drogues d'abus**, dans une **stratégie d'automédication**.

Dans le premier chapitre, nous avons apporté de nouveaux arguments en faveur de l'hypothèse glutamatergique du phénotype de type anxio-dépressif des rats PRS. En effet, nous avons montré que l'efficacité des antidépresseurs dans la correction des anomalies comportementales induites par le stress prénatal passait par une amélioration des déficits du système glutamatergique (**article 1**). Nous avons également étudié les rythmes circadiens de l'activité locomotrice, avec des perturbations du rythme circadien comme une caractéristique de la dépression, et nous avons mis en évidence que les rats PRS mâles et femelles présentaient tous deux des symptômes de type dépressif (**article 2**). Nous démontrons également que les hormones sexuelles jouent un rôle clé dans l'anxiété et la dépression.

Dans le chapitre 2, nous avons étendu la question du genre et des hormones sexuelles à la toxicomanie. Nous avons d'abord examiné si le stress prénatal pouvait influencer la sensibilité à la fois pour un psychostimulant (cocaïne) (**article 4**) et une récompense naturelle comme le chocolat **(article 3)**. Ce dernier point était important afin de démontrer qu'un stimulus naturel pourrait être une drogue. Dans cette partie, nous avons utilisé un paradigme de préférence de place conditionnée (CPP), qui consiste à coupler une drogue à un contexte environnemental particulier (signal ou « cue » contextuel). Nous avons montré que la préférence pour la cocaïne était renforcée chez les animaux PRS des deux sexes alors que le stress prénatal augmentait la préférence pour le chocolat chez les mâles uniquement.
Les hormones sexuelles modulaient la sensibilité induite par le stress prénatal. Les données comportementales ont trouvé des corrélats neuronaux ; en particulier, la sérotonine dans l'hypothalamus et la dopamine dans le noyau accumbens et le cortex préfrontal étaient affectées par le PRS, le sexe et les hormones sexuelles, d'une façon qui correspond bien avec une préférence pour le chocolat (**article 3**).

Un autre objectif de ce travail était de mieux établir un lien possible entre les troubles liés au stress et la propension à la consommation de drogues. Le modèle PRS, qui répond aux critères à la fois des deux troubles anxio-dépressifs et aussi addictifs, représente un très bon modèle pour répondre à cette question.

Ainsi, **dans le chapitre 3**, nous avons étudié l'effet de l'administration chronique de cocaïne sur le phénotype anxio-dépressif de rats contrôles et PRS mâles et femelles. Nous rapportons que la cocaïne est capable de corriger les symptômes anxio-dépressifs, de manière pathologie-dépendante. Le glutamate est apparu comme un régulateur clé des effets observés.

Dans l'ensemble, nos résultats indiquent que le modèle PRS est un bon modèle intégré de pathologies multiples liées entre elles, dans lequel la sensibilité aux drogues d'abus serait une stratégie d'automédication pour soulager les symptômes d'anxiété et de dépression, et dans lequel le genre, et en particulier les hormones sexuelles, sont des acteurs clés de la médiation de tous ces troubles comorbides liés au stress.

GENERAL INTRODUCTION

I. ADDICTION: GENERAL OVERVIEW

1. Definition

Even if often presented as an anglo-saxon neologism, the term addiction finds its terminology from the latin verb "*ad dicere*" (to give assent). From old Roman law to Middle Age, it meant that a person could be legally assigned to his creditor in case of debt. A slavery bond was so established toward a master, a kind of devotion (Véléa, 2005). It's also interesting to note that the origin of the word is the same as for "dictator" (Gregson and Efran, 2007). Addiction describes thus the alienating link that individual can establish with a product or a practice. Addiction is so a chronic psychiatric disorder which is characterized by compulsive drug use (Renthal and Nestler, 2008). Three main symptoms are defined in the Diagnostical and Statistical Manual of Mental Disorders, a manual that allows therapists to discriminate between pathologies in regard to symptomatology. Subjects suffering from drug addiction loose control toward the drug and i) have huge difficulties to limit their consumption, and moreover to stop it (loss of Control), ii) are always focusing on the ability to catch the drug and feel an irresistible want of the drug (Compulsive use) iii) haven't the capability to stop consuming the drug despite known negative effects (Continued use despite adverse consequences) (American Psychiatric Association, 1994, DSM IV), symptoms also referred to as the 3 C's. Patients also display important negative emotions (dysphoria, anxiety, irritability) when the access to the drug is limited (Koob and Le Moal, 1997). Addiction is distinct from dependence (Nestler, 2004), this last term further referring to physical aspects, namely physical pain, during withdrawal phases; dependence also occurs in case of chronic psychoactive medication, such as antidepressant treatment and beta-blockers, without any expression of addictive-like behavior. This distinction between these two considerations is now better established, with addiction meaning a loss of control (O'Brien et al., 2006).

Tolerance is also associated with drug addiction and is experienced when a specific dose taken previously to generate a certain kind of psychological state is no longer effective when taken subsequently. The individual thus has to increase the dose of the substance to maintain the same psychological effect of the drug (Siegel and Sapru, 2010).

Drug addiction is conceptualized as a disorder that progresses from impulsivity, associated with positive reinforcement (pleasure, gratification), to compulsivity, related to withdrawal symptoms (anxiety, depression, panic), which so represents a negative reinforcement. Impulse control disorders are characterized by an increasing tension and arousal before committing an impulsive act, with pleasure, gratification, or relief at the time of the act, and possible feelings of regret, guilt or self-reproach following the act. Conversely, in compulsive disorders, there are recurrent and persistent thoughts (obsessions) that cause anxiety and stress followed by the compulsive repetitive behavior (compulsion) that are aimed at preventing or reducing distress (DMS IV). As a subject moves from impulse control disorders to compulsivity, there is a shift from positive reinforcement for driving the motivated behavior to negative reinforcement driving the motivated behavior. The disease progresses in a spiraling/distress cycle comprising three stages: preoccupation/anticipation, binge/intoxication, withdrawal/negative affect (Koob and Le Moal, 1997).

Drug use cessation is indeed associated with strong withdrawal symptoms such as mood and sleep disorders, anxiety, depression, panic, unpleasant dreams (Sofuoglu et al., 2005; McGregor et al., 2005), and the severity of withdrawal symptoms can be predictive of high risk of relapse (Kenford et al., 2002). Cocaine withdrawal also induces anxiety- and depressive-like behaviors in rats (Perrine et al., 2008; Markou and Koob, 1991). The preclinical and clinical manifestations associated with abstinence period after chronic drug exposure can be alleviate by anxiolytic or antidepressant drugs (De Oliveira Citó et al., 2012; Harris, Altomare and Aston-Jones, 2001; Gawin et al., 1989; Mendelson and Mello, 1996).

According to the incentive-sensitization theory of addiction, proposed by Robinson and Berridge in 1993, psychomotor sensitization, a progressive and persistent increase in spontaneous locomotor activity observed following repeated and intermittent administration of psychostimulants, such as amphetamine (Robinson and Becker, 1986), would implicate long-term changes in the organization of brain systems that regulate reward and motivational phenomena. Thus, these circuits could become hypersensitive to the reinforcing properties of drugs and drug-associated stimuli, resulting in an excessive motivation and urge to consume these drugs (craving) (Robinson and Berridge, 1993; 2000; Kalivas et al., 1998). Repeated drug use sensitizes only the neural systems that mediate the motivational process of incentive salience (wanting), but not neural systems that mediate the pleasurable effects of drugs (liking) so that drugs can become addictive independently of their ability to produce a hedonic state (Wyvell and Berridge, 2000; Robinson and Berridge, 2008).

The sensitization phenomenon may last several months, even up to several years in rodents and humans, suggesting that psychostimulants persistently affect brain function (Nestler and Aghajanian, 1997; White and Kalivas, 1998). The incentive-sensitization or "wanting-liking" theory emphasizes the importance of drug-associated cues in addiction. Thus, in addicts, exposure to places, people or things (cues) that have been previously associated with drug-taking are predictive of a reward and cues acquire incentive motivational properties by themselves through Pavlovian learning, increasing the risk of relapse (Flagel, Akil and Robinson, 2009; Saunders and Robinson, 2013; Childress et al., 1993). In now classic studies, Pavlov (1927) indeed demonstrated that if a previously neutral stimulus (conditional stimulus, CS) reliably predicts the delivery of a reward (unconditional stimulus, US), over time the CS will come to elicit a conditional response (CR). Pavlov found that in hungry dogs, if the ticking of a metronome was paired with food delivery, the sound of the metronome itself (the CS) came to elicit salivation (the CR). Given that the dogs initially salivated unconditionally when presented with the US, Pavlov referred to the CS-elicited CR as a conditional reflex.

Moving from this evidence, more recent works show that when cues are associated with the delivery of rewards, animals come to quickly approach and engage the cue even if it is located at a distance from where the reward will be delivered. In these animals, the reward-predictive cue itself becomes attractive, eliciting approach towards it, presumably because it is attributed with incentive salience (Robinson et al., 2014; Flagel, Akil and Robinson, 2009). In the absence of drug stimulation, visual and/or auditory cues typically found in animal self-administration paradigms are capable of maintaining significant levels of operant behavior for extended periods of time and of reinstating the behavior after extinction (Caggiula et al., 2001, nicotine; Saunders and Robinson, 2010, cocaine; Krank, 2003, ethanol). It is thus important to take into account the importance of the environment and context in assessing the individual response to drugs.

The conception of addiction aforedescribed takes into consideration both the theoretical framework that addiction is a psychological construct, with drug use enabling people to cope with emotional and psychological states, and the medical model of addiction, which evokes neurobiological processes in addictive behavior (Clark M, 2011). Another model exists, the moral model of addiction, which is an unscientific perspective in which the addict is one who consciously and willingly decides to engage in the behavior on a regular basis. The addict is thus viewed as a free agent and is consequently culpable. Those who advance this model do

not accept that determining forces influence addictive behavior but rather propose that there is something morally wrong with people who use drugs heavily or gamble excessively. The moral model has little therapeutic value and implies that addicts should be punished rather than treated (Clark M, 2011).

2. Reinforcing stimuli

It is often considered that addiction refers to substances, such as cocaine, alcohol, amphetamines, that are psychoactive products, but there is mounting evidence that, among addiction, behavioral addiction, also referred to as nondrug addiction, exist (Holden, 2001). Thus, in DSM-V, a new category of behavioral addiction has been added; but, pathological gambling is the single disorder of this new class, while it was previously grouped with kleptomania, pyromania and trichotillomania in the category of "Impulse Control Disorders Not Elsewhere Classified". In this case, the negative consequence of the behavior is more of social, occupational or recreational concerns. Some members of the American Psychiatric Association (APA) argue that the APA's addictions category could be expanded even further to include "life-harming, compulsive" involvement with things like sex and food, which are classified in the DSM-V draft as separate "hypersexuality" and "binge eating" disorders (Peele, 2010). Peele also says that there is no habit that cannot become excessive, compulsive, life-endangering.

2.A. Epidemiology and statistics

During the five year period up to the end of 2010, the illicit drug use in the world remained stable at a rate between 3.4 and 6.6% the adult population (aged 15-64 years). However, between 10 and 13 % of consumers remain problematic users who have a drug addiction and/or drug use-associated disorders; prevalence of HIV (estimated at about 20%), hepatitis C (46.7 %) and hepatitis B (14.6 %) among drug users by injection still worsens the global burden of morbidity and especially, about 1 death per 100 is ascribed to illicit drug consumption . Opioids remain the major type of drug leading to requests for treatment in Asia and Europe, but also in Africa, North America and Oceania. Therapies for cocaine use concern mainly America and it is cannabis that creates the largest number of requests in Africa. In Asia, treatments for consumption of stimulants like amphetamine which are the most common needed. Globally, the two most widely used illicit drugs remain cannabis (annual prevalence ranging between 2.6 and 5.0%) and amphetamine-type stimulants,

exception made for ecstasy (between 0.3 and 1.2%). World annual prevalence of cocaine and opiates (opium and heroin) has remained stable with respective rates being between 0.3 and 0.4 and 0.3 % and 0.5 % of the population aged 15 to 64 year (United Nations Office on Drugs and Crime, 2012). The probability to develop an addiction is extremely variable depending on the substances consumed. The most addictive, both at physical level – involving withdrawal phenomenon-and at psychological level, which implies an irrepressible desire, are by far tobacco and heroin. Near a smoker per three would develop a tobacco addiction, 23% of people consuming heroin will develop an addiction for the drug, 17% for cocaine, 15% for alcohol, 11% for other stimulants, 9% for cannabis and psychotropics/analgesics, 5% for hallucinogenic drugs (Anthony, Warner, and Kessler, 1994). An estimated 24% of US adolescent and adult household residents have had an opportunity to use cocaine, as compared to 14% for hallucinogens, 5% for heroin, and 51% for marijuana (Van Etten and Anthony, 1999), a rank-ordering that is also seen in estimates for the prevalence of use of these drugs (Anthony et al., 1989).

2.B. Drugs: focus on cocaine

2.B.1. Response to drugs

Reward system

The brain reward system has evolved to respond to natural reward essential for the survival moving from the Darwin theory of evolution (Wise and Bozarth, 1981; Bozarth, 1994). Indeed, brain reward systems serve to direct the organism's behavior toward goals that are normally beneficial and promote survival of the individual (e.g., food and water intake) or the species (e.g., reproductive behavior) as suggested by Troland's concept of beneception (Troland, 1928).

In 1954, James Olds and Peter Milner, working at McGill University in Canada first discovered that direct electrical stimulation of the brain can be powerfully rewarding…by accident. The two researchers were studying brain stimulation of areas involved in alertness and learning to assess if it was possible to train rats to avoid some part of a cage associated with stimulation. One of the electrodes was implanted by mistake in the septum, leading the rat to return to places where it received stimulation. Olds and Milner thus showed that direct stimulation of brain areas was able to activate reward circuits in such an important manner that rats died because they did not feel anymore the need to eat or drink nor to engage in reproductive behaviors when presented to a putative mating partner. They carried out their

pioneering experiments which discovered that rats would repeatedly press levers to receive tiny jolts of current injected through electrodes implanted deep within their brains (Olds and Milner, 1954). Especially when this brain stimulation was targeted at certain areas of the brain in the region of the septum and nucleus accumbens, the rats would repeatedly press the lever - even up to 2000 times per hour (Olds, 1956). These powerful findings suggest that Olds and Milner had discovered the pleasure center in the brain (Higgins and George, 2009).

Drugs of abuse are hijacking the brain being able to directly stimulate the brain reward system, with very quick and strong effect (Teresi, 2011).

Drugs of abuse

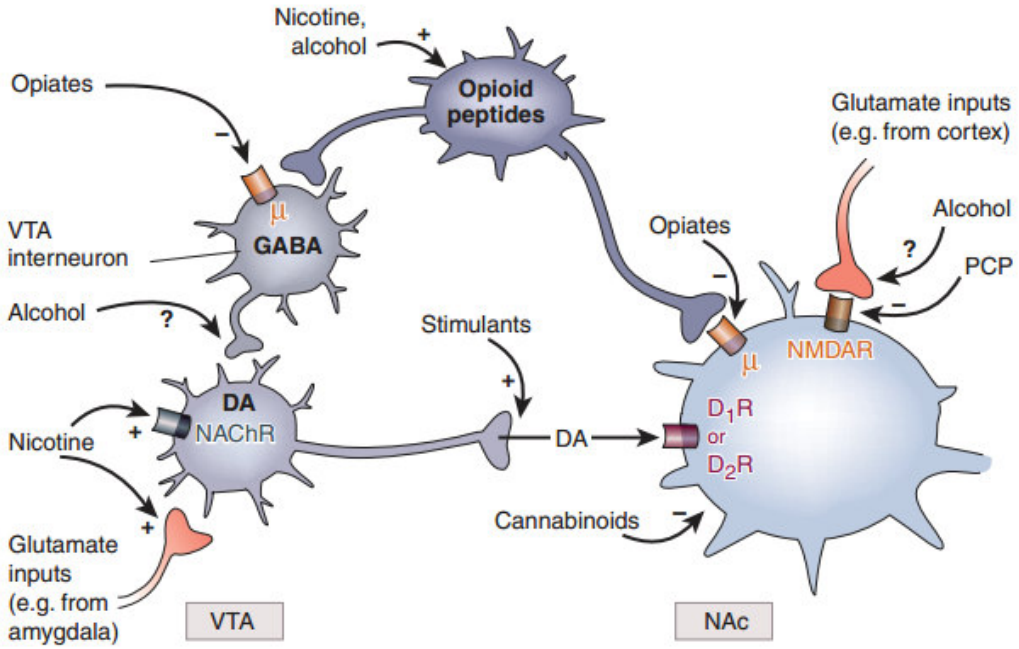


Fig 1- Actions of drugs in reward circuits.

VTA DA neurons project to the NAc. Different interneurons interact with VTA and NAc neurons. The rewarding properties of opiates are mediated by μ opiate receptors in two locations in brain reward circuits. VTA DA neurons are tonically inhibited by GABAergic interneurons that express μ opiate receptors. Opiates acutely inhibit these interneurons thus disinhibiting the DA projection neurons which then release DA in the NAc and other terminal fields. In addition, there are μ opiate receptors expressed by NAc and dorsal striatal neurons. Opiates can stimulate these receptors directly and produce reward in a DA independent manner. Nicotine, acting on nicotine acetylcholine receptors (NAChRs) in the VTA, causes DA release. Ethyl alcohol, acting on GABAa receptors in the VTA, can also cause DA release. Phencyclidine (PCP), which blocks the NMDA glutamate receptor channel and cannabinoids acting via CB1 cannabinoid receptors in the VTA (not shown), also produce D release. Cannabinoids, alcohol and PCP can also act directly on the NAc, PCP. From Hyman, Malenka and Nestler, 2006.

Drugs thus mimic or enhance the actions of endogenous chemical messengers in the brain, including dopamine (DA), serotonin (5-HT), acetylcholine, glutamate, gamma-aminobutyric acid (GABA) and various peptides (Wise, 1996; Bardo, 1998; Fig. 1). The focus for the neurobiological mechanism for the positive-reinforcing effects of drugs of abuse has been the mesocorticolimbic DA system, which projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), olfactory tubercle, frontal cortex and amygdala (Koob and Le Moal, 2001; Fig. 2). Selective destruction of the mesocorticolimbic dopamine system with the neurotoxin 6-hydroxydopamine (6-OHDA) produced extinction-like responding in cocaine or nicotine self-administration as reflected in a significant and long-lasting reduction in responding over days (Roberts and Koob, 1982; Corrigall et al., 1992).

For cocaine, amphetamine, and nicotine, the facilitation of DA neurotransmission in the mesocorticolimbic DA system, and in particular in the NAc, appears to be critical for the acute reinforcing actions of these drugs (Di Chiara and Imperato, 1988). Multiple DA receptors including D1, D2, and D3 have been implicated in this reinforcing action (Koob and Le Moal, 1997). A common molecular underpinnings are shown for several drugs of abuse with upregulation of the cAMP pathway in the NAc; and, many groups reported numerous, additional adaptations in the VTA – NAc pathway, some of which are also common to several drugs of abuse. These include alterations in levels of G-protein subunits, tyrosine hydroxylase (the rate limiting enzyme in dopamine biosynthesis), glutamate receptors and neuropeptide systems (Ortiz et al., 1995; Nestler, 1992; Striplin and Kalivas, 1992; Hanson et al., 1992; Fitzgerald et al., 1996; Carlezon and Nestler, 2002).

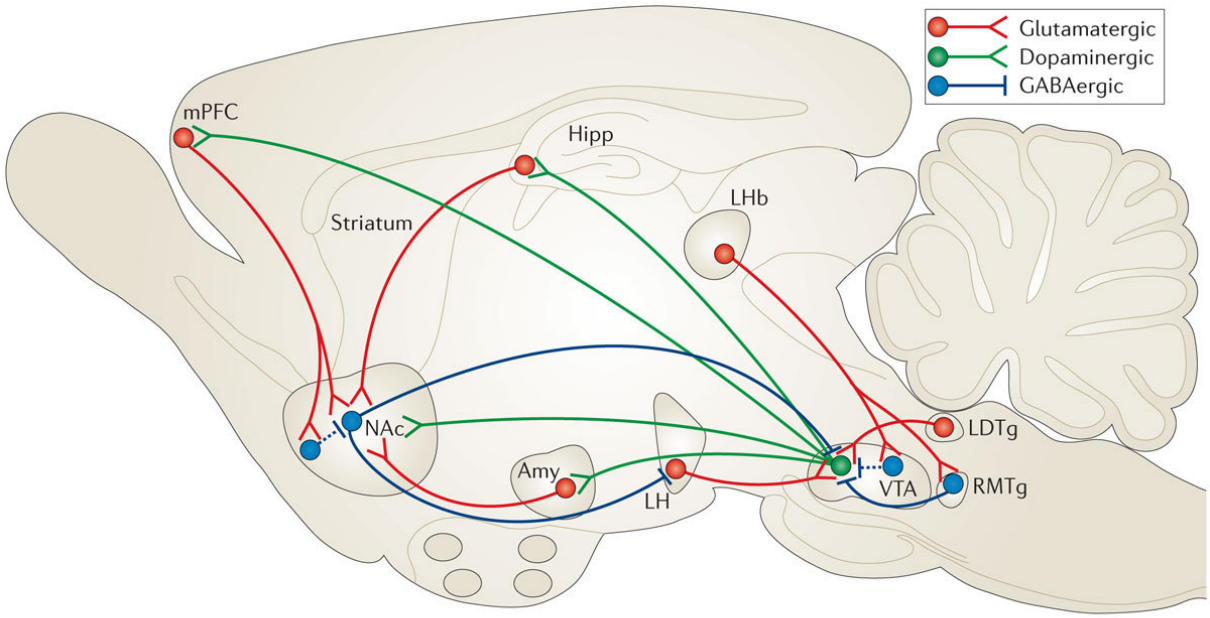


Fig 2- A simplified schematic of the major dopaminergic, glutamatergic and GABAergic connections to and from the ventral tegmental area (VTA) and nucleus accumbens (NAc) in the rodent brain.

The primary reward circuit includes dopaminergic projections from the VTA to the NAc, which release DA in response to reward-related stimuli (and in some cases, aversion-related stimuli). There are also GABAergic projections from the NAc to the VTA, projections through the direct pathway (mediated by D1-type medium spiny neurons (MSNs)) directly innervate the VTA, whereas projections through the indirect pathway (mediated by D2-type MSNs) innervate the VTA *via* intervening GABAergic neurons in the ventral palladium (not shown). The NAc receives dense innervations from glutamatergic monosynaptic circuits from the medial prefrontal cortex (mPFC), hippocampus (Hipp) and amygdala (Amy), as well as other regions. The VTA receives such inputs from the lateral dorsal tegmentum (LDTg), lateral habenula (LHb) and lateral hypothalamus (LH), as well as both GABAergic and glutamatergic connections from the extended amygdala (not shown). These various glutamatergic inputs control aspects of reward-related perception and memory. The dashed lines indicate inhibitory projections. The glutamatergic circuit from the LH to the VTA is also mediated by orexin (not shown). RTMg: rostromedial tegmentum. From Russo and Nestler, 2013.

Among the multiple dopaminergic terminal regions examined, the nucleus accumbens (NAc) stands out as a particularly important participant in reward-related motivated behavior, although other structures have also been implicated. In terms of circuitry, the NAc is well positioned to integrate limbic inputs associated with memory, affect, motivation and goal-directed motor activity (Kalivas, Sorg and Hooks, 1993; Ikemoto and Panksepp, 1999). The NAc receives heavy afferent projections from the VTA, as well as a variety of cortical and subcortical structures including the prefrontal cortex, the hippocampus, and the basolateral amygdala (Zahm and Brog, 1992; Brog et al., 1993).

2.B.2. The example of cocaine

Origine

Cocaine is an alkaloid derived from the leaf of Erythroxylon coca (Dackis and O'Brien, 2001) Early Spanish explorers noticed how the native people of South America were able to fight off fatigue by chewing on coca leaves. A medical account of the coca plant was published in 1569 (Belzman, 2010). The German chemist Friedrich Gaedcke was the first in 1855 to have distilled coca leaves and obtained a substance that he called erythroxyline. In 1860, Albert Niemann isolated the active cocaine from the coca leaf and described the anesthetic action of the drug when it was put on his tongue. 5 years later, Wilhelm Lossen found the chemical formula (Clarac and Ternaux, 2008).

There are several ways of consumption: intranasal (snorted) in 63% of cases, inhaled (smoked, in case of crack) in 31% of cases or injected intravenously in 3% of cases. These last two routes of administration are quite similar in terms of effects (speed of action, physiological and psychotropic effects, powerful action potential). Intranasal cocaine is the most consumed, especially in integrated classes and festive situations. The effects begin 3-5 minutes after taking and last for 30 minutes (Karila et al., 2014).

Cocaine produces a ''rush'' or "high" of intense euphoria that is well outside the normal range of human experience. The power of cocaine reward is demonstrated by the fact that the drug is readily self-administered to the point of death by laboratory animals and preferred over feeding and mating opportunity (Dackis and Gold, 1985). Cocaine users feel wellbeing, disinhibition, increased energy, increased self-esteem, racing thoughts, logorrhea, reduced hunger, increased libido under the action of the drug; these psychological effects are sometimes accompanied by neurovegetative signs (tachycardia, pupillary dilation, increased blood pressure and increased temperature) (Karila et al., 2014).

Originally, cocaine was made popular by the nineties and appeared also in literature, with the famous fictional detective Sherlock Holmes injecting cocaine to relieve boredom (Golden and Peterson, 2009). There is no doubt that the use that made the Indians for centuries will remain the main mode of cocaine use: it will be assessed in all cases where it is a priority issue to increase the physical capacities of the organism for a relatively short time and keep in reserve forces to mobilize in case of need - especially when external circumstances exclude any possibility of rest or food normally required for performing provided exercises. Cocaine was used a local anesthetic and for a variety of illnesses and alcohol or morphine addictions. Unfortunately, many of patients went on to become addicted to cocaine (Kolb and Whishaw, 2002). In the past, cocaine was widely used in the preparation of drinks and cocktails made with wine, proposed as invigorating drinks. It is the origin of the brand name Coca- cola, simply because, when developed in 1886 by John Pemberton the drink contained both cocaine and caffeine. Angelo Mariani, in the early 1880s produced a "medicinal" wine, called Mariani wine, which contained 11% alcohol and 6.5 mg of cocaine in every ounce. Cocaine was removed from Coca Cola in 1906 (but it still has the caffeine). The Harrison Narcotic Act in 1914 made cocaine illegal. Finally, in 1985, crack cocaine was introduced, cheaper than cocaine, while providing a more intense experience, and rapidly became a major drug problem (Golden and Peterson, 2009).

Mode of action

One particular part of the limbic system, the NAc, seems, as described previously, to be the most important site of the cocaine high (Nestler, 2005). Cocaine is shown to be a potent inhibitor of all three monoamine transporters, those for DA, 5-HT, and norepinephrine, and thereby potentiates monoaminergic transmission (Koob and Nestler, 1997). But, although cocaine also inhibits the transporters for the other neurotransmitter chemicals, its actions on the DA system is generally thought to be most important (Nestler, 2005).When stimulated by DA, cells in the NAc produce feelings of pleasure and satisfaction. By artificially causing a buildup of DA in the NAc, as mentioned above, cocaine yields enormously powerful feelings of pleasure. The amount of DA connecting to receptors in the NAc after a dose of cocaine can exceed the amounts associated with natural activities, producing pleasure greater than that which follows thirst-quenching or sex. In fact, some laboratory animals, if given a choice, will ignore food and keep taking cocaine until they starve (Nestler, 2005). Increases in NAc DA release during intravenous cocaine self-administration have been confirmed using *in vivo* microdialysis (Hurd et al., 1989, Weiss et al., 1992). It has also been shown that DA receptor antagonists can decrease the reinforcing effects of cocaine in self-administration in rats and block Conditioned Place Preference (CPP, a test which will described in further details afterwards in 5.2) for this drug (Hachimine et al., 2014; Pruitt, Bolanos and McDougall, 1995). All three DA receptor subtypes have been implicated in the reinforcing actions of cocaine as measured by intravenous self-administration including the D1 (Maldonado et al., 1993), D2 (Caine et al., 2002; Bergman, Kamien and Spealman, 1990), and D3 receptors (Peng et al., 2009; Caine and Koob, 1993). DA D1 receptors antagonists also block the place conditioning produced by amphetamine (Liao, Chang and Wang, 1998; Beninger, 1992, Leone and Di Chiara, 1987). DA lesions and microinjection of DA antagonists into the mesocorticolimbic DA system block cocaine reinforcement (Maldonado et al., 1993; Callahan, De La Garza and Cunningham, 1997). Data with knockout mice also provide key insights into the role of DA in the rewarding effects of drugs of abuse (Koob and Volkow, 2010). Genetically altered mice homozygous with a lack of the DA D1 receptor do not self-administer cocaine (Caine et al., 2007). Besides, transgenic animals that express a functional DAT that binds cocaine, is efficient as DA reuptake carrier, but is insensitive to the drug, don’t show cocaine reward as measured by conditioned place preference (Chen et al., 2000). These results support the hypothesis of a crucial role of the DAT in cocaine’s reinforcing effects.

The glutamatergic system, with glutamate being the primary excitatory neurotransmitter in the brain and a mediator of the synaptic plasticity required for organisms to adapt to environmental changes (Abraham, 2008), appears critically involved in drug addiction (reviewed by Kalivas et al., 2009; Wolf et al., 2004). Activity-dependent long-term depression (LTD) and long-term potentiation (LTP) of synaptic transmission are the two principal forms of synaptic plasticity that permit strengthening (LTP) or weakening (LTD) of synapses in an interplay that allows the refinement of neuronal circuits necessary to adapt behavior to an ever-changing environment (Malenka and Bear, 2004). Transition to addition in cocaine- addict rats is indeed related to a long-lasting impairment in LTD, which results in a persistent deficit in synaptic plasticity (Kasanetz et al., 2010), and cocaine increases glutamate in the NAc (Pierce et al., 1996).

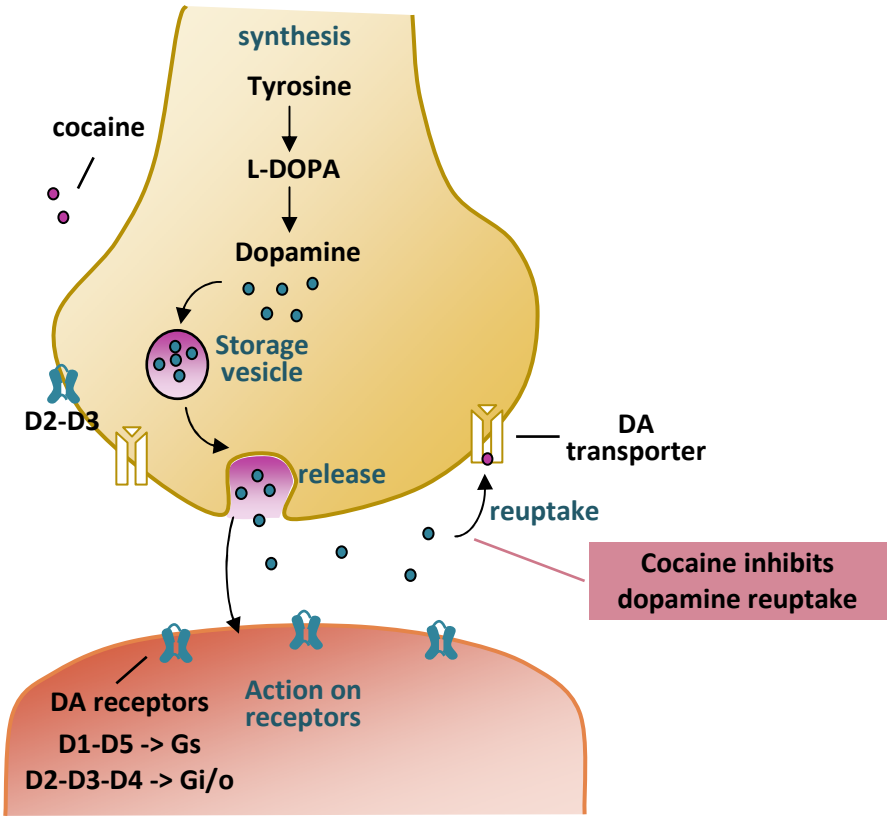


Fig 3- Cocaine interaction with the dopaminergic synapse.

Under normal conditions, DA neuron firing results in DA-filled vesicles to fuse with the presynaptic membrane and subsequently DA is released into the synaptic cleft. Once in the synapse, DA can bind to post-synaptic DA receptors and then is removed from extracellular space by the dopamine transporter (DAT). Cocaine increases levels of DA by binding to the DAT and thereby inhibiting DA uptake back into the terminal. As DA uptake is reduced, levels accumulate in the synapse and DA has a greater opportunity to bind to dopamine receptors. DA receptors are coupled to G proteins. Gs stimulate adenylyl cyclase activity. In contrast, Gi/o are inhibitory G protein and decrease the formation of cAMP. Adapted from Freberg, 2009; España and Jones, 2013.

Epigenetic regulation

Administration of psychostimulants such as cocaine causes the induction of proteins Fos and Zif 268 in the striatum, the NAC and the prefrontal cortex, the target structures of dopaminergic and nigrostriatal mesocorticolimbic systems (Graybiel, Moratalla, and Robertson, 1990; Hope et al., 1992; Jaber et al., 1995; Unal et al., 2009). Mice deficient for Zif-268 protein do not exhibit conditioned place preference for cocaine and behavioral sensitization to drugs is also reduced, suggesting an important role of this factor in the rewarding and stimulating effects of cocaine (Valjent et al., 2006). Another transcription factor has been identified in the NAc following repeated drug exposure: ΔfosB (Nestler, 2001). While cocaine affects several transcription factors, its effects on ΔFosB are the most long-lasting. ΔFosB is naturally present in small quantities in the cells of the NAc, but chronic cocaine exposure causes it to accumulate to high levels (Nestler, Barrot, and Self, 2001). Researchers believe ΔFosB may constitute an important molecular "switch" in the transition from drug abuse to addiction (Nestler, 2005).

Epigenetic mechanisms, such as histone acetylation, phosphorylation and methylation in the NAc and other brain areas suggests that such modifications might be involved in regulating behavioral responses to cocaine and other drugs of abuse (Reviewed by Renthal and Nestler, 2008; see also Fig .4). Indeed, the first evidence for this came from studies that demonstrated that the pharmacological and genetic manipulation of certain histone deacetylases (HDACs) in the NAc alters levels of histone acetylation *in vivo* and profoundly affects behavioral sensitivity to cocaine (Kumar et al., 2005). In the conditioned place preference test, in which an animal learns to associate the rewarding effects of cocaine with a specific environment, either systemic administration of sodium butyrate or trichostatin A, both non-specific HDAC inhibitors, significantly potentiates the rewarding effects of cocaine (Kumar et al., 2005). Delivery of the more specific HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) directly into the NAc is sufficient to increase cocaine reward (Renthal et al., 2007). On the contrary, in a study conducted in rats and not in mice, a reduction in cocaine self-administration was found when animals were treated with HDAC inhibitors (Romieu et al., 2008).

Consistent with the hypothesis that increased histone acetylation potentiates behavioral sensitivity to cocaine, mice that are deficient in histone acetyl transferase (HAT) exhibit reduced histone acetylation and reduced sensitivity to cocaine (Levine et al., 2005). Similarly, reducing histone acetylation in the NAc by virally overexpressing certain HDACs significantly decreases cocaine place conditioning (Renthal et al., 2007).

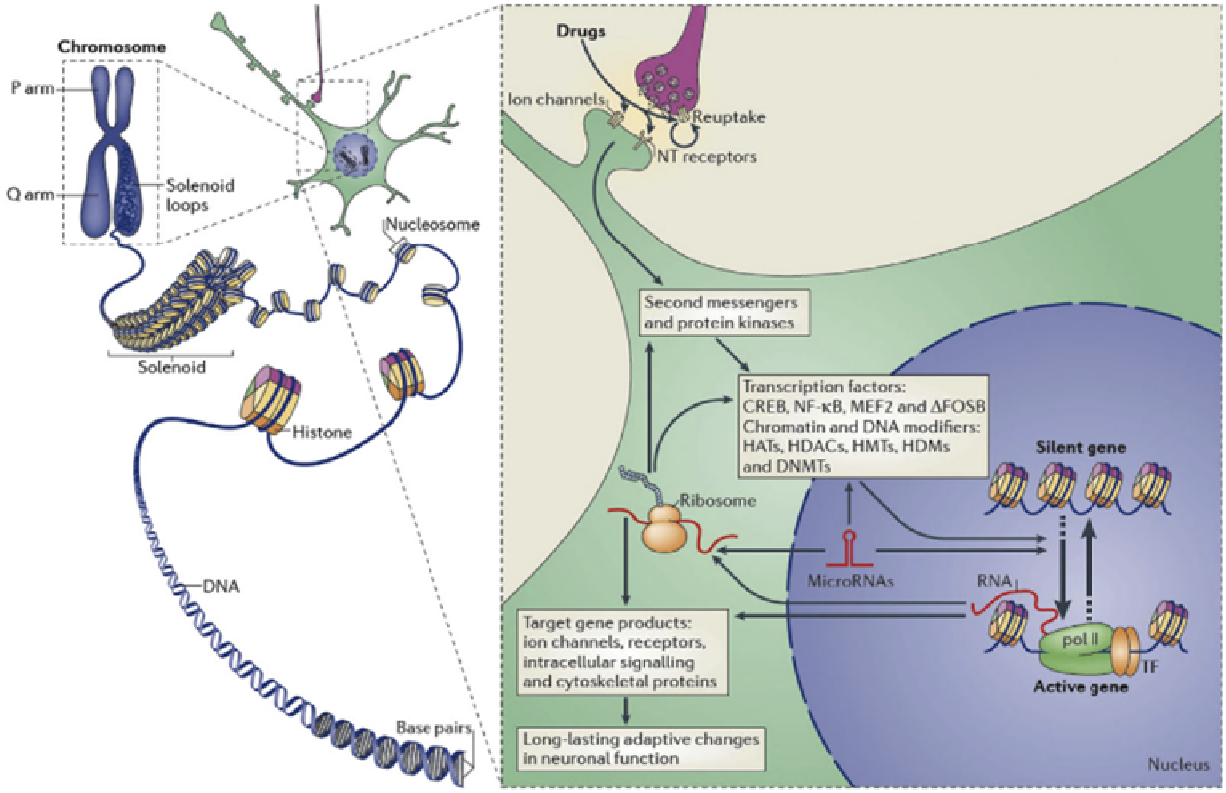


Fig 4- Epigenetic regulation by drugs of abuse.

In eukaryotic cells, DNA wraps around histone octamers to form nucleosomes, which are then further organized and condensed to form chromosomes (right). Unraveling compacted chromatin makes the DNA of a specific gene accessible to the transcriptional machinery. Drugs of abuse (left) act through synaptic targets to alter intracellular signaling cascades, which leads to the activation or inhibition of transcription factors and of many other nuclear proteins; the detailed mechanisms involved in the latter remain poorly understood. This leads to the induction or repression of particular genes, including those for noncoding RNAs; altered expression of some of these genes can in turn further regulate gene transcription. It is hypothesized that some of these drug-induced changes at the chromatin level are extremely stable and thereby underlie the long-lasting behaviors that define addiction. CREB, cAMP response element binding protein; DNMTs, DNA methyltransferases; HATs, histone acetyltransferases; HDACs, histone deacetylases; HDMs, histone demethylases; HMTs, histone methyltransferases; MEF2, myocyte enhancing factor-2; NFκB, nuclear factor κB; pol II, RNA polymerase II. From Nestler, 2014.

3. Natural-behavioral rewards

As previously described, among addiction, there are growing evidence for behavioral addiction, with some behaviors like sport, gambling, sex, shopping that share similarities with the action of drugs of abuse and lead to the activation of neurotransmitters involved in the response to psychoactive drugs (Holden C, 2001).

As far as the prevalence of obesity over the last decades is increasing, not only in American countries, but worldwide (Finucane et al., 2011), and as there is a strong correlation between overweighting and development of type 2 diabetes, cardiovascular disease, osteoarthritis, sleep and mental disorders (Stettler and Shelly, 2009), obesity is a very important health problem, and it becomes fundamental to understand what mechanisms underlie these troubles. The idea that food addiction could exist brings important explanations for eating disorders such as “binge eating” or hyperphagia and consequent obesity (Benton, 2010). If foods are considered, it is the hedonic aspect of the stimulus that leads to loss of control. Interestingly, the touch, temperature, texture, paste, smell and appearance of food, which contributes to its palatability, can activate the same neuronal circuits as addictive drugs. Thus, the nucleus tractus solitarius (NTS) relays informations related to food palatability, coming from chemosensory neurons in the oral cavity and afferents from the gastrointestinal tract. NTS neurons so project to brain regions involved in drug reward processing, like regulation of reinforcing properties of drug of abuse and development of drug dependence, namely NAc, amygdala and BNST (Kenny, 2011).

Chronic sucrose consumption in rats increases ΔFosB expression in NAc and an overexpression of ΔFosB in the NAc is able to increase sucrose intake. The same facilitating effect of ΔFosB was observed concerning sexual behavior. The fact that a key marker induced by strong drugs of abuse, such as psychostimulants, can also be overexpressed by natural rewards like food or sex, gives further evidence in favor of the reality of compulsive behavior toward these natural rewards (Wallace et al., 2008). Of note, an association, “Overeaters Anonymous” exits, like Alcoholics Anonymous. Compulsive overeating certainly has the look of an addiction that can dominate a person’s life. There is also biochemical evidence suggesting a kinship (Holden, 2001). In 2008, Volkow et al. found that, in a group of compulsive overeaters, DA receptor availability was lower, an anomaly also seen in drug addicts, and also described by this Team for metamphetamine (Volkow et al., 2001). DA deficiency in obese individuals may perpetuate pathological eating as a mean to compensate

for decreased activation of these circuits. A common mechanism targeted by foods and drugs of abuse is the DA system. Feeding, like psychostimulants injection, increases DA release in the NAc, and the reduction of D2 DA receptors revealed by brain imaging in people suffering from obesity, which is related to body mass index, is close to the one observed in drug-addicted subjects (Volkow and Wise, 2005; Wang et al., 2001). Rats under a daily intermittent sucrose drinking schedule escalate sucrose intake over 21 days of test and show a huge consumption of sucrose during the first hour of access, which can be referred to as a "binge". Interestingly, control rats on daily intermittent chow don't eat more on day 21 than they do on day 1, suggesting the importance of the tastiness of the food for the escalation of consumption. Sucrose bingeing is also associated with an increase in DA release in NAc in the intermittent sucrose group, always found on day 21, whereas the increase in DA release seen on day 1 in intermittent chow or daily *ad libitum* sucrose groups was not seen anymore after 21 days. Escalation of palatable food intake and the concomitant enhancement of DA release reinforce the hypothesis that food may cause dependence almost like drugs of abuse (Rada et al., 2005).

Bulimia, which is characterized by bingeing and vomiting, also looks a lot like an addiction (Davis and Claridge, 1998).

Anorexia, which involves self-imposed starvation, due to the fear of becoming fat, is characterized by anxious avoidance of weight gain, compulsive control of eating and weight, being more a disorder of "anxiety" and "impulse regulation" than of "feeding". By contrast, symptomatology in bulimia nervosa implies recurrent binge eating and frantic compensatory behaviors (such as vomiting, laxative misuse or compulsive exercise) (Steiger, 2004), a compulsive behavior followed by guilt and remorse in patients (Colles, Dixon and O'Brien, 2008). As with drug addictions, bulimic behavior is initially voluntary but is transformed into a compulsion because of changes that it wreaks on the nervous system. Bulimia clearly affects reward centers: patients become increasingly depressed and anxious before episodes; immediately following, they uniformly report a pleasant "afterglow" (Faris et al., 2008; Carei et al., 2010). A novel hypothesis that bulimia dysregulates the vagal nerve, which regulates heart and lungs as well as the vomiting impulse was proposed (Faris et al., 2000). It is suggested that a binge-purge episode then brings the vagal nerve back to its normal role. This retraining of the vagal nerve also has long-term effects on the brain's reward circuitry, as suggested by the fact that bulimics have a high relapse rate and are very hard to help once they've been at it for a few years (Frank, 2013).

Interestingly, in rats who have experienced intensive cocaine seeking, and developed a high vulnerability to the drug, sucrose is able to surpass cocaine, and when they have the choice between a lever which delivers cocaine and another which delivers a sweetness solution, rats prefer sweet (Lenoir et al., 2007, Fig. 5); rats also display a greater attraction to and preference for sweet versus drug cues (Madsen and Ahmed, 2014).

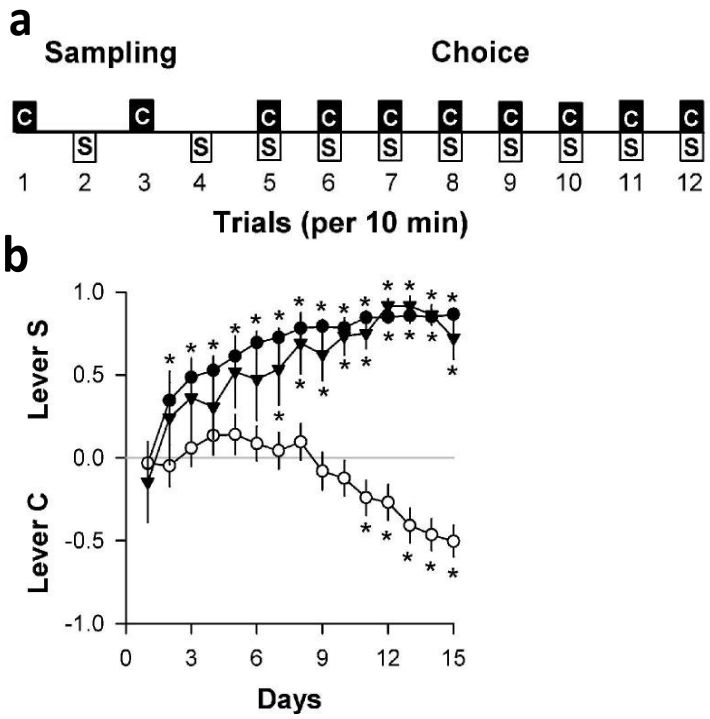


Fig 5- Rats prefer sucrose over cocaine.

a, Schematic representation of the choice procedure. Each choice session was constituted of 12 discrete trials, spaced by 10 min, and divided into two successive phases, sampling (4 trials) followed by choice (8 trials). S, saccharin-associated lever; C, cocaine-associated lever. b, Choice between levers C and S (mean±SEM) across reward conditions and as a function of time (open circle: S-/C+ condition; closed triangle: S+/C- condition; closed circle: S+/C+ condition). The horizontal gray line at 0 indicates the indifference level. Values above 0 indicate a preference for lever S while values below 0 indicate a preference for lever C. *, different from the indifference level ($P<0.05$, *t*-test). *, different from lever S ($P<0.05$, Fisher's LSD test after a two-way analysis of variance). From Lenoir et al., 2007.

In a very recent study conducted in the Connecticut College, J Schroeder (2013) and his students have fed rats with Oreos cookies as a food that is palatable to humans and contributes to obesity in the same way that cocaine is pleasurable and addictive to humans. On one side of a maze, hungry rats received Oreos and on the other, rice cakes as a control ("Just like humans, rats don't seem to get much pleasure out of eating them," Schroeder said.).

Then, rats had the option of spending time on either side of the maze and the time spent on the side where rats were typically fed Oreos was measured. The team compared the results of the Oreo and rice cake test with results from rats that were given an injection of cocaine or morphine, known addictive substances, on one side of the maze and a shot of saline on the other. The research showed that the rats conditioned with Oreos spent as much time on the "drug" side of the maze as the rats conditioned with cocaine or morphine. Immunohistochemistry was then used to measure the expression c-Fos in the NAc. Oreos were found to significantly activate more neurons than cocaine or morphine. This correlated well with behavioral results and supported the hypothesis that high-fat/ high-sugar foods are addictive.

Nearly 15 % of men and 30% of women admit to compulsive chocolate cravings. Among substances composing chocolate, caffeine and theobromine could have reinforcing effect, but these substances would be left in too little to really have an effect. The same conclusion is made concerning phenylethylamine, a substance related to amphetamines. Finally, recently, a neurotransmitter produced naturally by the brain, anandamide has been isolated in chocolate. Neuronal receptors for anandamide are also those which bind THC, the active ingredient of cannabis. Anandamide may therefore contribute to a sense of well-being reported by people hooked chocolate (although more than 30 pounds of chocolate should be ingested to be comparable to a dose of cannabis effects). Anyway, many scientists agree that the addiction to chocolate could simply be due to its good taste that causes a sensation of intense pleasure we want to repeat (Bruinsma and Taren, 1999; Nasser et al., 2011).

To bring further arguments in favor of the suggestion that food can be addictive; a drug which is efficient in decreasing alcohol dependence, craving, consumption and withdrawal symptoms shows also an ability to decrease the reinforcing effect of foods. Thus, the gamma-hydroxybutyric acid (GHB) analogue N-(4-trifluoromethylbenzyl)-4-methoxybutanamide (GET73) preference of rats both to sweetened corn flakes and cafeteria diet in the conditioned place preference test (Ottani et al., 2007). In mice, deep brain stimulation (DPS) of the nucleus accumbens, a procedure currently under investigation in humans for the treatment of major depression, obsessive-compulsive disorder and substance abuse (Schlaepfer and Bewernick, 2013; Tsai et al., 2014; Pierce and Vassoler, 2013), attenuates binge eating, an effect mediated by the dopamine D2 receptor, as D2R antagonism by raclopride blocks the beneficial effect of DPS. A decrease in body weight and an improve in glucose tolerance are also demonstrated with nucleus accumbens DBS (Halpern et al., 2013).

The epidemic of illness related to overweight and obesity is a public health problem of great significance. The hypothesis for food addiction is attractive because it provides an explanatory context by which the obesity epidemic and its myriad adverse health consequences can be understood in the same framework as the epidemic of drug addiction (Ifland et al., 2009).

4. Interindividual vulnerability

Addiction is not simply the consequence of a intense and prolonged consumption of a drug but to the intrinsic vulnerability of the user. Thus, among people who display an extensive drug use, only a few percentage of individuals will develop drug addiction (Anthony, Warner, and Kessler, 1994; Reboussin and Anthony, 2006). The same pattern is obtained in animal models: in rats, addiction-like behavior is present only in a small proportion of subjects using cocaine and is highly predictive of relapse after withdrawal (Deroche-Gamonet, Belin and Piazza, 2004). Moreover, when given a choice, the minority of rats displaying a cocaine-preferring profile would be homologous to the minority of cocaine users with a diagnosis of cocaine addiction (Ahmed, 2010). Thereby, all individuals will not be equal for transition from drug use to abuse (Brady, Sonne and Lydiard, 1993; Piazza et al., 1996) and several factors will intervene in shaping vulnerability.

4.A. Age

Adolescence has been defined as a critical window for the onset of a first consumption with a drug, linked to the need to seek novel experiences, a higher risk-taking, involving sometimes use of substances like cannabis, alcohol or nicotine; and the age at the first diagnosis of substance use disorders shows a peak between 15 and 18 years old (NIAAA, 2003). Interestingly, a higher vulnerability to drugs of abuse during adolescence has also been demonstrated in animal models. Thus, cocaine conditioned place preference was established at lower doses in adolescent rats (Zakharova, Wade and Izenwasser, 2009) as well as enhanced cocaine-induced locomotor activity (Catlow and Kirstein, 2005; Hollis et al., 2012).

4.B. Individual factors

Epidemiological studies have revealed a striking association between the sensation/novelty seeking trait, as well as impulsivity in substance use disorders (Kreek et al., 2005).

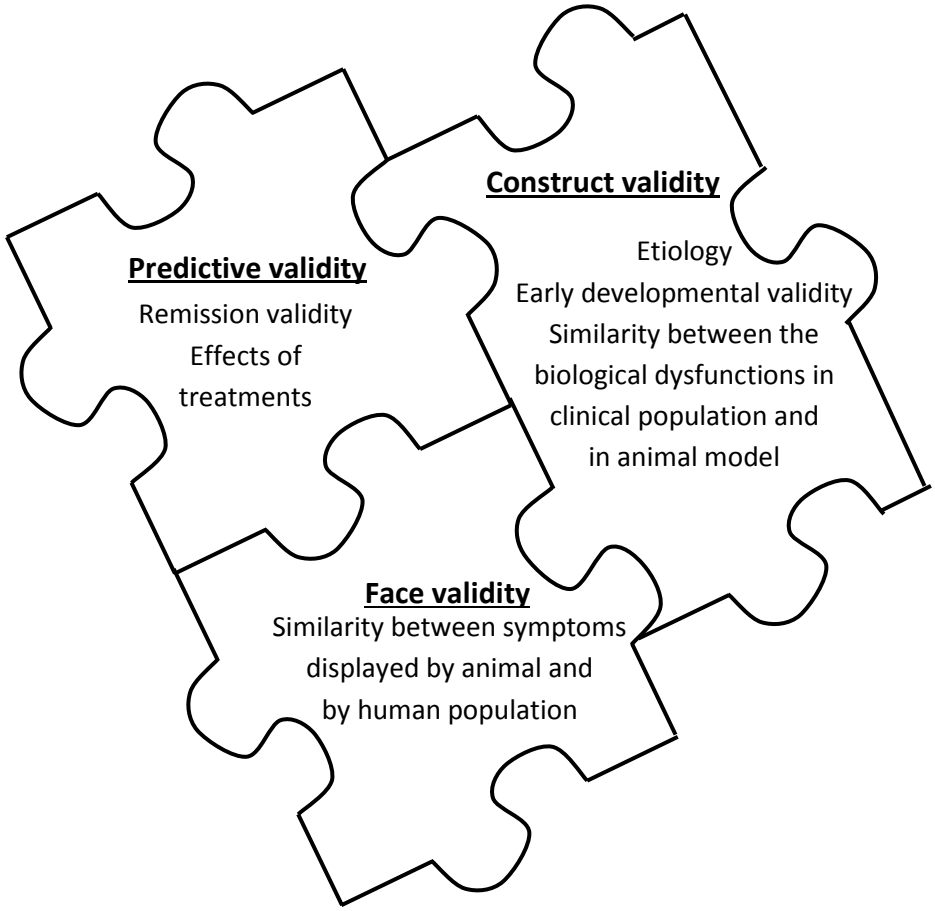
Thus, sensation/novelty seekers are more prone to experience addictive drugs, akin to various risky activities (Zuckerman, Ball and Black, 1990; Franques, 2003; Buckman et al., 2009) and impulsivity is a predictive trait for drug addiction (Verdejo-García, Lawrence and Clark, 2008; Moeller et al., 2001). The sensation/novelty-seeking trait can be studied in rodents both by high locomotor reactivity to a new inescapable environment (high-responder (HR) phenotype; Kabbaj, 2006; Dellu et al., 1996; Blanchard et al., 2009), and high propensity to visit a new environment in a free-choice procedure, ie, novelty-induced conditioned place preference (CPP); high-novelty-preferring (HNP) phenotype (Bardo et al., 1996; Cain, Saucier and Bardo, 2005). Both traits are dependent on the dopaminergic system (Dellu et al., 1996; Bardo et al., 1996). HR rats display decreased anxiety in exploring novelty (Kabbaj et al., 2000) and are more vulnerable than low-responders (LR) in their propensity to acquire drug self-administration (Piazza et al., 1989, 1990a, 2000), and in their response to the locomotor effect of the drug (Deroche et al., 1993). Rats identified as high novelty seekers display a higher vulnerability to express CPP for amphetamine (Klebaur and Bardo, 1999) and novelty preference but not locomotor reactivity to novelty predisposes animals to cocaine addiction (Belin et al., 2011). High impulsivity (assessed by operant 5-choice serial reaction time task (5-CSRTT) is also predictive for cocaine addiction (Belin et al., 2008; Anker et al., 2009, Molander et al., 2011). In this paradigm, animals are trained to enter a food magazine to initiate a trial. After an intertrial interval (ITI) of 5 s had elapsed, a brief light stimulus is randomly presented in one of five apertures. Following a nose-poke in this aperture (‘correct’ response), animals are rewarded with the delivery of a food pellet in the magazine. A nose-poke response in any of the adjacent apertures (‘incorrect response’), or a failure to respond within 5 s after the onset of the stimulus (‘omission’), results in no food delivery and a time-out period with the house light extinguished for 5 s. Nose-pokes made during the ITI, that is, before the onset of the stimulus (or ‘premature responses’) are recorded as a measure of impulsivity, and results in a 5 s time-out and no food reward (Besson et al., 2013, from Robbins, 2002).

5. Animal models of addiction

5.A. Interest and validity of animal model

Animal models are of considerable help in the understanding of some mechanisms involved in the pathophysiology of human disorders and in the development of therapeutic treatments.

Nevertheless, to be considered relevant for a translational view, animal models must fulfill a multidimensional set of criteria of validity, first elaborated by Willner (1984), and reviewed by Belzong and Lemoine (2011). Face validity corresponds to the extent of similarity between the model and the disorder examined, with, as wide as possible, a range of symptoms and signs similar to the features seen clinically. Construct validity is seen as an attempt to establish a theoretical rationale of animal models both at the level of a similarity of the behavioral and/or cognitive dysfunctional processes and at the level of a similarity of the etiology of the abnormalities seen; also, the link between these two levels is important: the theoretical account of the disordered behavior in the model and the theoretical account of the disorder itself have to be brought into alignment. Predictive validity is a criterion that relies on a human-animal correlation of therapeutic outcomes, with treatment modalities effective in reversing human pathological symptoms able to reverse changes seen in animals.



5.B. Experimental paradigms

In the understanding of neuronal aspects involved in the propensity of individual to consume drugs, progressively lose control toward the drug and don't manage to stop taken the drug despite awareness on adverse consequences on health and/or social life, and in the perspective to develop efficient therapies, animal models are essential (Vanderschuren and Ahmed, 2013).

DSM-IV criterion	Behavioral equivalent
Tolerance, withdrawal	Tolerance, escalation of drug use
Using more than intended	Impaired control, neurocognitive deficits
Difficulty restricting	Resistance to extinction
Great deal of time spent	Increased motivation for drug
Other activities given up	Drug preference over nondrug rewards
Continued use despite problems	Resistance to punishment

Fig 6- Appearance of DSM-IV criteria in animal studies of drug addiction.
Adapted from Vanderschuren and Ahmed (2013).

Drug addiction can be studied in animal models according to several paradigms that mimics symptoms of addiction described in humans (Deroche-Gamonet, Belin and Piazza, 2004).

We have previously described the three main symptoms of addiction reported by the DSM. These symptoms can be studied in animal models of self-administrations: (i) The subject has difficulty stopping drug use or limiting drug intake. The persistence of drug seeking is assessed during a period of signaled drug non-availability. The daily self-administration (SA) session includes three 40-min "drug periods" that are separated by two 15-min "no-drug periods." During the drug periods, a standard fixed ration (FR) reinforcement schedule is used: a fixed number of nose-pokes results in an infusion of drug. During the no-drug periods, nose-pokes have no effect. The two different periods of drug availability are signaled by a change in the illumination of the SA chamber. (ii) The subject has an extremely high motivation to take the drug, with activities focused on its procurement and consumption. A progressive-ratio schedule is used: the number of responses required to receive one infusion of drug (i.e., the ratio of responding to reward) is increased progressively within the SA session.

The maximal amount of work that the animal will perform before cessation of responding, referred to as the breaking point, is considered a reliable index of the motivation for the drug (Richardson and Roberts, 1996). (iii) Substance use is continued despite its harmful consequences. The persistence of the animals responding for a drug when drug delivery is associated with a punishment is measured.
During these sessions, nose-pokes on the standard FR schedule results in the delivery of both the drug and an electric shock. This shock punishment is signaled by a new cue light that is turned on at the time of the first nose-poke and off after the delivery of the shock.

The propensity of animals to relapse to drug seeking ("reinstatement" procedure, Shaham et al., 2003) is also an important parameter, as, in humans, the most predictable outcome of a first diagnosis of addiction is a 90% chance of relapse to drug use even after long periods of withdrawal (DeJong, 1994). After a period of withdrawal that followed period of SA, rats are exposed to stimuli known to induce relapse in humans, such as small amounts of the abused drug or a conditioned stimulus associated with drug taking. These challenges induce high levels of responding (reinstatement) on the device previously associated with drug delivery.

When using models of sustained drug use, it is then important to set drug availability within a range that best mimics the human condition. In humans, by definition, drug access during the recreational phase is quite restricted, principally because of the large space occupied by competing activities (Piazza and Deroche-Gamonet, 2013), suggesting the importance of models in which animals are offered the choice between drugs and alternative activity (Ahmed, Lenoir and Guillem, 2013). It has also been shown that escalation of drug consumption, as an important hallmark of transition from drug use to addiction (Ahmed, 2011), was one of the first features of addiction demonstrated in rats with extended access to cocaine, but not in control rats with limited access to the drug (Ahmed and Koob, 1998). Specifically, with extended access to cocaine self-administration, cocaine self-administration gradually increased across days, while, with more limited drug access, it remained remarkably stable, even after several months of testing (Ahmed and Koob, 1999; Ahmed, 2005). Moreover, rats with extended access to cocaine will be more prone to continue to seek drug during punishment by footshock (Ahmed, 2011).

As we have seen at the beginning of the chapter, environmental cues are very important in drug abuse. In that context, the conditioned place preference (CPP) paradigm has been widely used as a preclinical behavioral model to study rewarding effects of drugs and investigate

mechanisms underlying substance dependence and evaluation of new drugs for its therapy. The test assesses the animal's ability to associate drug-induced effects with environmental cues, thus providing an important model of neuroadaptations associated with addiction process and measures the reinforcing properties of drugs and food as much as reveals the role of contextual memories in the reinforcement process (Prus, James and Rosecrans, 2009). The basic characteristics of this task involve the association of a particular environment with drug treatment, followed by the association of a different environment with the absence of the drug (i.e., the drug's vehicle). A common variation of this design consists of a three-compartment chamber with the outer compartments being designed to have different characteristics (e.g., white vs. black walls, pine vs. corn bedding and horizontal grid vs. cross-grid flooring). The middle compartment has no special characteristics and is not paired with a drug, and the gates between the compartments can be opened to allow an animal to pass freely between them. During training, animal is given an injection of a drug with potentially rewarding properties, and is then placed into one of the outer compartments for several minutes. On the following day, the rat is injected with the drug's vehicle and then placed in the opposite compartment. Generally, these daily conditioning sessions alternate between drug and vehicle for 2 or 3 days each. Afterward, a test session is conducted, which consists of placing the animal in the center compartment and then, after opening the gates to both of the outer compartments, recording the time the animal spends in each of the outer compartments during the session. A conditioned place preference (CPP) is found if the animals spend significantly more time in the drug-paired compartment versus the vehicle-paired compartment. Typically, drugs of abuse, such as cocaine, produce CPP and in drug-dependent animals, withdrawal generally produces conditioned place aversion. Because the CPP paradigm generally provides a reliable indicator for studying the rewarding effects of drugs that require relatively little training compared to self-administration paradigm, the CPP paradigm has been commonly used in conjunction with standard neuroscience techniques to elucidate the subjective effects of drugs (Zakharova, Wade and Izenwasser, 2009).

Throughout the thesis, we have chosen to work with this particular paradigm.

II. STRESS AS A FACTOR INVOLVED IN PSYCHOPATHOLOGIES

1. What is stress?

The term of “stress” has been defined by Hans Selye (1936) as the nonspecific response of the body to any demand, with a stressor being an agent that produces stress at any time.

Selye expended thus the works of Cannon (1932) that showed that an organism is preparing itself to deal with a treat by increasing blood pressure, mobilizing glucose, increasing respiration and inhibiting unnecessary energy consuming processes *via* the release of epinephrine (also known as adrenaline).

The idea that all fundamental processes necessary for the maintenance and life of the tissue elements are maintained in an equilibrium independently of the stability of the environment was early introduced by Claude Bernard (1813-1878) and this original concept of “Milieu intérieur” (Bernard, 1865) was later developed by Cannon as the homeostasis concept. Homeostasis (from greek: "hómoios", "similar" and “*stásis*, "to remain”) — is the property of a system in which variables are regulated so that internal conditions remain stable and relatively constant in spite of environmental variations and disturbances. Both the mind/brain and the body are endowed with a multitude of automatic mechanisms of feedback-inhibition that counteract influences tending toward disequilibrium.

The general adaptation syndrome (GAS) or stress syndrom described by Selye represents the chronologic development of the response to stressors when their action is prolonged. The GAS hypothesis involves the hypothalamic – pituitary – adrenal (HPA) axis and the release of glucocorticoids hormones, and consists in three phases: the alarm reaction, corresponding to the physiological activation of the HPA axis and the sympathetic nervous system [SNS] in preparation to deal with a threat), a resistance stage (the period following the initial reaction to the threat whereby the body mediates ongoing stress and attempts to return to steady - state levels), and an exhaustion stage, when a prolonged stress response overexerts the body’s defense systems, thus draining it of its reserve resources and leading to illness.

A central construct of Seyle’s integrative model of stress was the notion of homeostasis. In order to clarify the inherent ambiguity in the term “homeostasis” by distinguishing between systems that are essential for life (homeostasis) and those that maintain these systems in balance, the term “allostasis” has been coined from the Greek allo, which means "variable;" thus, "remaining stable by being variable" (Sterling and Eyer, 1988; Klein et al., 2004) with

the concept of allostasis referring to the superordinate system by which stability is achieved *via* change (McEwen, 2005). The concept of allostasis is a fundamental process through which organisms actively adjust to both predictable and unpredictable events. Allostatic regulation reflects, at least partly, cephalic involvement in primary regulatory events, in that it is anticipatory to systemic physiological regulation (Sterling and Eyer, 1988; Schulkin, 2003). Thus, the concept of homeostasis rather applies to a limited number of physiologic variables (end points), such as pH, body temperature, glucose levels and oxygen tension, that are truly essential for life and that are therefore maintained within a narrow range of their respective set-points. Allostatic load refers to the cumulative cost to the body for being forced to adapt to adverse psychosocial or physical situations and maintain allostasis, and it represents either the presence of too much stress or the inefficient operation of the stress hormone response system, which must be turned on and then turned off again after the stressful situation is over, with allostatic overload being a state in which serious pathophysiology can occur. Using the balance between energy input and expenditure as the basis for applying the concept of allostasis, two types of allostatic overload have been proposed (McEwen and Wingfield 2003).

The conceptual framework of an allostatic overload, the wear and tear on the body and brain that result from being “stressed out” has created a need to know how to improve the efficiency of the adaptive response to stressors while minimizing overactivity of the same systems, since such overactivity results in many of the common diseases of modern life. This framework has also helped to demystify the biology of stress by emphasizing the protective as well as the damaging effects of the body’s attempts to cope with the challenges known as stressors (McEwen, 2005).

While stress has often a negative connotation, stress response can be “good” and results in cognitive and behavioral responses generating pleasant sensations, and also adequate processes to regain homeostasis (McEwen, 2002). At the core of an acute stress response is the initiation of the fight - or - flight response, which is characterized by the rapid activation of the sympathetic nervous system, which leads to the release of noradrenaline from widely distributed synapses and adrenaline from the adrenal medulla, that serve as the first response to prepare the body for the energy resources it will require (Thiel and Dretsch, 2011; De Klöet, Joëls and Holsboer, 2005). Upon activation, epinephrine and norepinephrine are released from the adrenal medulla into circulation and induce a cascade of physiological effects including increased respiration rate, increased heart rate, dilation of skeletal muscle

blood vessels, glycogen to glucose conversion, and vasoconstriction of digestive and reproductive organ blood vessels. These changes serve to selectively increase blood flow and oxygen/glucose availability to brain tissues and skeletal muscles that require energy to prepare for action (McCarty, 2000).

When aversively perceived encounters cannot be controlled by fight or flight, strategies involving the hypothalamo–pituitary–adrenocortical (HPA) axis will play a vital role in adaptation of the organism to the homeostatic challenge (Engelmann, Landgraf and Wotjak, 2004). The HPA axis is activated by both internal and external signals; when a situation is perceived as stressful, the brain activates many neuronal circuits to adapt to the demand (De Klöet, Joëls and Holsboer, 2005). In most vertebrates there is a pronounced circadian rhythm in glucocorticoid secretion (Chung, Son and Kim, 2011), with peaks corresponding to the onset of the active phase of the diurnal cycle awakening to ready physiological systems for activity and metabolic challenge (Kalsbeek et al., 2012; Keller-Wood and Dallman, 1984). The diurnal rise in glucocorticoids is allowed through a reduction in inhibitory tone from the suprachiasmatic nuclei (SCN) of the hypothalamus projecting to the paraventricular hypothalamic nucleus (PVN) (Szafaraczyk et al., 1983; Kalsbeek et al., 1996) coupled with an increased adrenal sensitivity to adrenocorticotropic hormone (ACTH) through autonomic inputs, thereby enhancing corticosterone secretion (Oster et al., 2006); and lesions of the SCN flatten the corticosteroid rhythm to levels intermediate those of the circadian peak and nadir (Cascio, Shinsako and Dallman, 1987; Moore and Eichler, 1972).

The HPA axis is controlled by a discrete set of hypophysiotrophic neurons in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN). These neurons synthesize and secrete corticotropin releasing hormone (CRH), the primary secretagog for ACTH , that causes the release of pituitary adrenocorticotrophic hormone (ACTH) by binding to CRH-R1 receptors (Aguilera et al., 2004), as well as a cocktail of other factors (e.g., arginine vasopressin (AVP)) that modulate ACTH release. Secretagogs travel by way of the hypophysial portal veins to access anterior pituitary corticotropes, which then stimulate release of ACTH into the systemic circulation. Glucocorticoids (cortisol in human, corticosterone in mouse and rat) are then synthesized and released upon binding of ACTH in the adrenal cortex (Antoni, 1986; Whitnall, 1993; De Klöet et al., 1987; De Klöet, Joëls and Holsboer, 2005). Negative feedback by circulating glucocorticoid levels keep basal and stress reactive secretion of the HPA axis under tight control. Two receptors, the mineralocorticoid

receptor (MR) and the glucocorticoid receptor (GR), mediate the actions of glucocorticoids in the central nervous system (Reul and De Klöet, 1986; Sapolsky et al., 2000).

Basal levels of CRH and ACTH secretion are controlled by the actions of the high affinity type I corticosteroid receptor MR. In contrast, elevations that occur following stress are controlled predominantly by activation of the type II corticosteroid receptor GR. Since the GR has a lesser affinity for corticosteroids than does the MR (Reul and De Klöet, 1985), this allows it to respond to elevated corticosteroid levels to reduce the stress-induced secretions of CRH and ACTH (De Klöet et al., 1998). Given that glucocorticoids can affect many behaviors, a negative feedback mechanism is essential for keeping the balance of the HPA axis activity during both basal conditions and in response to stress (Ratka et al., 1989). CRH is the main regulator of anterior pituitary ACTH secretion; however, the role of vasopressin (AVP), as a co-secretagog has been also recognized (Herman, Wiegand and Watson, 1990). CRH neurons of the PVN receive afferences relayed by glutamatergic (Ziegler and Herman, 2000) and more especially GABAergics interneurons localized within the immediate environment of the PVN, which can be named the peri-PVN area. The CRH neurons controlling the HPA activity largely express glutamate and GABA receptors. Thus, the activation of these CRH neurons can occur after glutamatergic stimulation but it seems more obvious that a disinhibition, exerted by an inhibition of the GABAergic interneurons projecting on the CRH neurons, takes larger place in this activation. On the contrary, the inhibition of these CRH neurons would pass mainly by an activation of these GABAergic interneurons (Herman et al., 2002).

Organisms are often confronted with prolonged challenges that require continuous adaptation. In this case, the brain has to adjust HPA axis function to cope with repeated stress while maintaining responsiveness to new potential threats. If the repeated stressor is sufficiently benign, the HPA axis response will undergo substantial attenuation with repeated stimulation, and many of the more pronounced physiological changes seen with initial stress. Notably, the maintained or sensitized response often occurs against a backdrop of elevated glucocorticoids, and is thus considered an active process that overcomes feedback. The latter mechanism was extensively characterized by Dallman and colleagues, and is referred to as facilitation (Akana et al., 1992). If stressors are sufficiently intense or unpredictable (as observed in stress regimens providing randomized exposure to heterotypic stressors), the animal is not able to successfully habituate. In these regimens, weight loss and HPA axis hyper-activity are maintained over time (Herman, Adams and Prewitt, 1995) and significant dysfunction encountered, as measured by both aberrant neural changes (McEwen, 1999) and development

of depression-like behaviors (Willner, 2005). These types of regimen provide parallels to numerous human conditions and are used to model disease states and test potential palliative interventions (Herman, 2011). It has also been shown that inhibition of GRs and MRs altered information processing in rats (De Klöet, Oitzl and Joëls, 1999).

A dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis is one of the most commonly described alterations that correlate with symptoms of mood disorders and other neuropsychiatric diseases. Therefore, an understanding of the neurobiological mechanisms controlling the HPA axis is important for deciphering potential changes that can impact the risk for such disorders (Fernández-Guasti et al., 2012).

Epidemiological data show that there is a link between situations of chronic stress and alterations of mental health (burnout) (Ahola et al., 2006), mood disorders (anxiety, depression) (Godin et al., 2005; Melchior et al., 2007; Netterstrom et al., 2008; Bonde, 2008; Siegrist, 2008), sleep disturbances (Akerstedt, 2006; Armon et al., 2008), disorders of consumption behaviors (substances, alcohol) (Head, Stansfeld and Siegrist, 2004; Siegrist and Rödel, 2006). To date, there is not a single causal mechanism linking stress and mental health, but the HPA axis seems to be largely involved. The hyperactivity of this system in response to stress is certainly driven by hypersecretion of CRH in the hypothalamus.

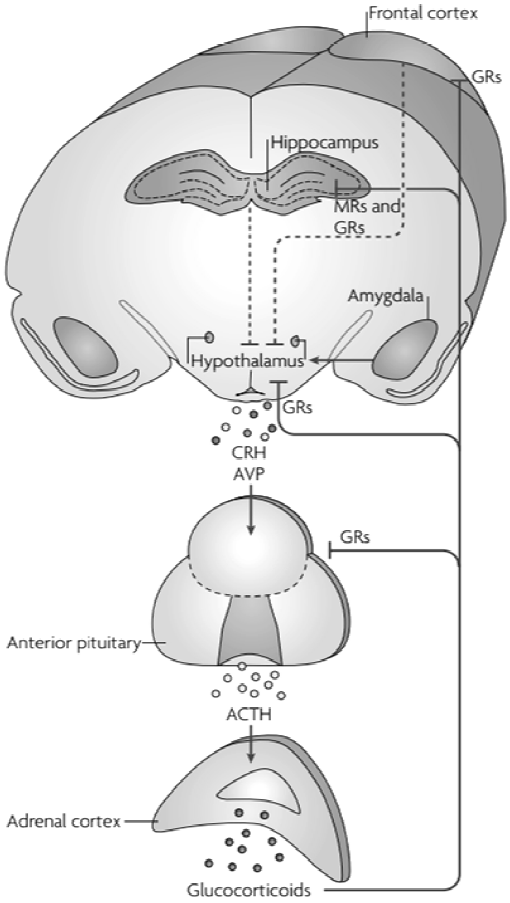


Fig 7- The stress system.

When the brain detects a threat, a coordinated physiological response involving autonomic, neuroendocrine, metabolic and immune system components is activated. A key system in the stress response that has been extensively studied is the hypothalamus-pituitary-adrenal (HPA) axis. Neurons in the medial parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). This triggers the subsequent secretion of adrenocortico-tropic hormone (ACTH) from the pituitary gland, leading to the production of glucocorticoids by the adrenal cortex. In addition, the adrenal medulla releases catecholamines (adrenaline and noradrenaline) (not shown, but mentioned in the text). The responsiveness of the HPA axis to stress is in part determined by the ability of glucocorticoids to regulate ACTH and CRH release by binding to two corticosteroid receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Following activation of the system, and once the perceived stressor has subsided, feedback loops are triggered at various levels of the system (that is, from the adrenal gland to the hypothalamus and other brain regions such as the hippocampus and the frontal cortex) in order to shut the HPA axis down and return to a set homeostatic point. By contrast, the amygdala, which is involved in fear processing, activates the HPA axis in order to set in motion the stress response that is necessary to deal with the challenge. Not shown are the other major systems and factors that respond to stress, including the autonomic nervous system, the inflammatory cytokines and the metabolic hormones. All of these are affected by HPA activity and, in turn, affect HPA function, and they are also implicated in the pathophysiological changes that occur in response to chronic stress, from early experiences into adult life. Adapted from Lupien et al., 2009.

2. Stress-related disorders

Although many individuals experiencing stressful events do not develop pathologies, stress seems to be a provoking factor in those individuals with particular vulnerability, determined by genetic factors or earlier experience (McEwen, 2008). The chronic hyper-activation of the hypothalamus-pituitary-adrenal (HPA) axis can be determined by multiple factors including genetic and environmental factors. The perinatal life, infancy, childhood and adolescence are periods of increased plasticity for the stress system and are, therefore, particularly sensitive to stressors. Adverse stressors during these critical periods of life may affect behaviors and physiologic functions, such as growth, metabolism, reproduction and the inflammatory/immune response (Seckl, 2008; Fig. 8). These environmental triggers or stressors may not have a transient, but rather a permanent effect on the organism. Barker (1999) has emphasized how adult vulnerability to disease may be programmed during the foetal stage. Indeed, non-genetic factors that could act early in life to organise or imprint permanently physiological systems are known as perinatal **programming**.

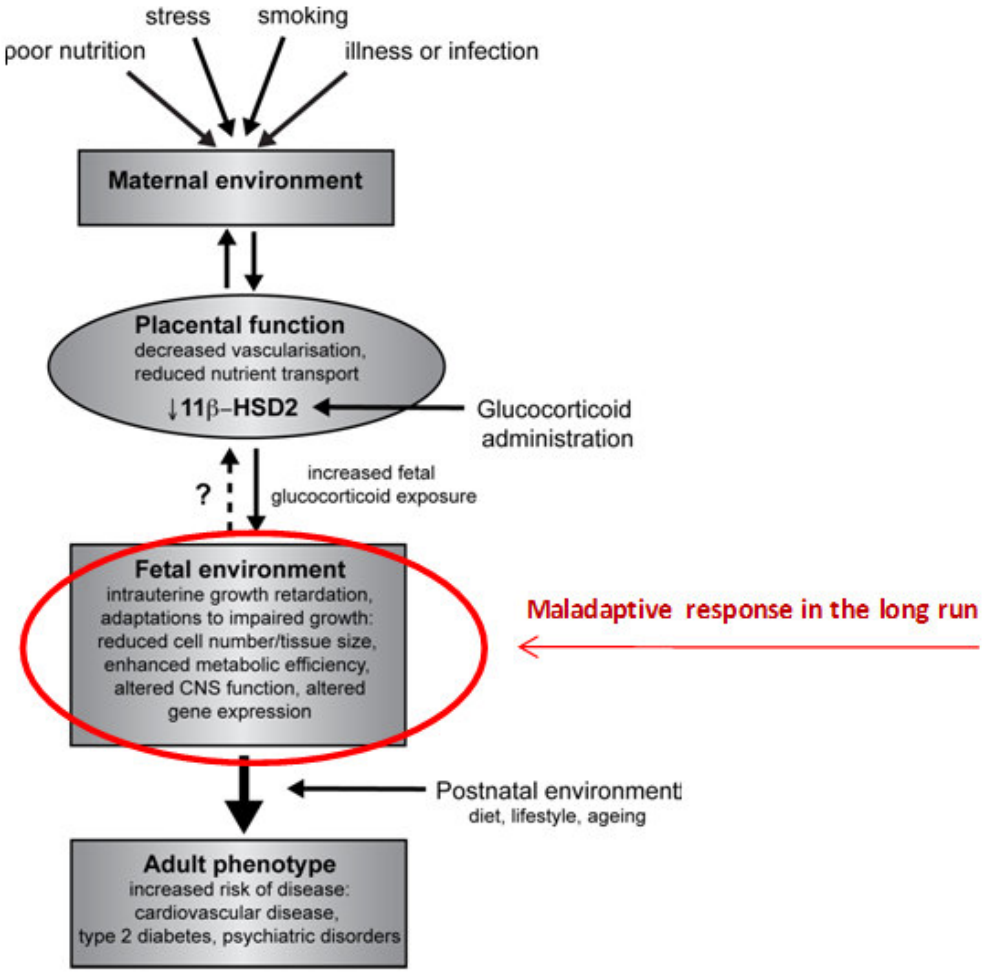


Fig 8- Developmental foetal programming. Adapted from Cottrel and Seckl, 2009.

2.A. Anxiety, depression

Evidence suggests that stressful life events are of great importance in the etiology of number of psychiatric and mood disorders, as well as mental illness. Thus, an important prevalence of psychiatric troubles has been found in case of abuse, maltreatment and neglect during childhood (Keyes et al., 2012). Loss (decrease of well-being linked to real (death or parental separation) or realistically imagined loss of a person, material possessions, health, respect, employment), humiliation (feeling devalued in relation to others or to a core sense of self, usually with an element of rejection or a sense of role failure), entrapment (severe events and difficulties of at least 6 months duration that is expected to persist or get worse, with little or no possibility that a resolution can be achieved) and danger (level of potential future loss, including the chance that a given traumatic event will recur or reflecting a possible sequence of circumstances in which the full threat or dire outcome has yet to be realized) are predictive of the onset of major depression (MD) and general anxiety syndrome (GAS) (Kendler, Karkowski and Prescott, 1999; Kendler et al., 2003). Women with a history of childhood sexual abuse present an increased risk for developing psychopathologies, such as MD, GAS, alcohol and other drug dependence (Kendler et al., 2000) and maternal prenatal or postnatal stress, mother adversity in relationship with partners (change, separation, quality) and poverty are risk factors for the development of anxious/depressive disorders (Phillips et al., 2005), with sustained alterations of the HPA axis observed in case of traumatic separation endured in childhood (Bloch et al., 2007). Moreover, adversity in childhood is predictive of increased response to a stressor at adulthood (Mclaughlin et al., 2010). In addition, chronic unpredictable or immobilization stress in rats induces emotional and cognitive deficits (Bondi et al., 2008; Wood et al., 2008).

It has also been shown that excessive activation of the HPA axis is also responsible for the increase of the basal secretion of glucocorticoids observed in some depressed patients, probably due to a deficiency of the negative feedback of the axis (Pariante and Lightman, 2008). Circadian abnormalities such as phase advances of the cortisol rhythm and reductions in the amplitude of the rhythms are also mentioned as a link between chronic stress and depression (Souêtre et al., 1989; Keller et al., 2006; Wirz-Justice, 2006). Recently, the hypothesis of an impairment of neural plasticity (structural and functional) due to chronic stress and leading to depression was refined paving the way for new therapies (Fuchs et al., 2004; Pittenger and Duman, 2008). Prolonged glucocorticoid exposure seems also involved in hippocampus atrophies described by Sapolski since 2000 in depressed patients (Sapolski, 2000).

Alike, memory deficits encountered in depressive patients could be explained by attenuation of long-term potentiation under chronic stress (De Klöet, 2004).

2.B. Addiction

Stressful life experiences are also predictive of vulnerability to drug abuse (Keyes et al., 2012). Exposure to stressful life experiences, such as maltreatment, catastrophic events, occupational or financial difficulties leads to an enhanced risk of developing alcohol use disorders (reviewed by Keyes, Hatzenbuehler and Hasin, 2011).

Several preclinical studies have shown the ability of stressors to alter the acquisition of drug self-administration in rats (Goeders, 2002; Piazza and Le Moal, 1998). The acquisition of amphetamine and cocaine self-administration is enhanced in rats exposed to tail pinch (Piazza et al., 1990b), social defeat (Haney et al., 1995; Tidey and Miczek, 1997; Kabbaj et al., 2001) or neonatal isolation (Kosten et al., 2000). Early life stress, in the form of maternal separation also enhances behavioral sensitization to morphine (Kalinichev, Easterling and Holtzman, 2002). Exposure to electric footshock also increases the subsequent reinforcing efficacy of heroin (Shaham and Stewart, 1994) and morphine in rats (Will, Watkins and Maier, 1998). The ability of stressors to facilitate the acquisition of drug self-administration may therefore result from a similar sensitization phenomenon, perhaps involving dopamine (Goeders, 1997; Piazza and Le Moal, 1998). Although exposure to the stressor itself may be aversive, the net result is reflected as an increased sensitivity to the drug. Therefore, if certain individuals are more sensitive to stress (Piazza and Le Moal, 1998) and/or if they find themselves in an environment where they do not feel that they have adequate control over this stress (Levine, 2000), then these individuals may be more likely to engage in substance abuse.

The HPA axis upregulation and the resulting increased release of glucocorticoids appears responsible for the enhanced sensitivity to the effects of drugs of abuse, and the increase in self-administration under stressful conditions (Maccari et al., 1991; Rougé-Pont et al., 1998).

Thus, glucocorticoids enhance the release of DA, especially in the NAc and progressively impair glucocorticoid negative feedback by decreasing the number of central corticoid receptors in the hippocampus. Stress reactivity is a reliable predictor for vulnerability to abuse. Naïve rats selected for an initial high reactivity in a mild stressful novel environment present a higher reactivity of the stress axis and are more likely to self-administer drugs (Piazza and Le Moal, 1996, 1997; Piazza et al., 1991, 1996).

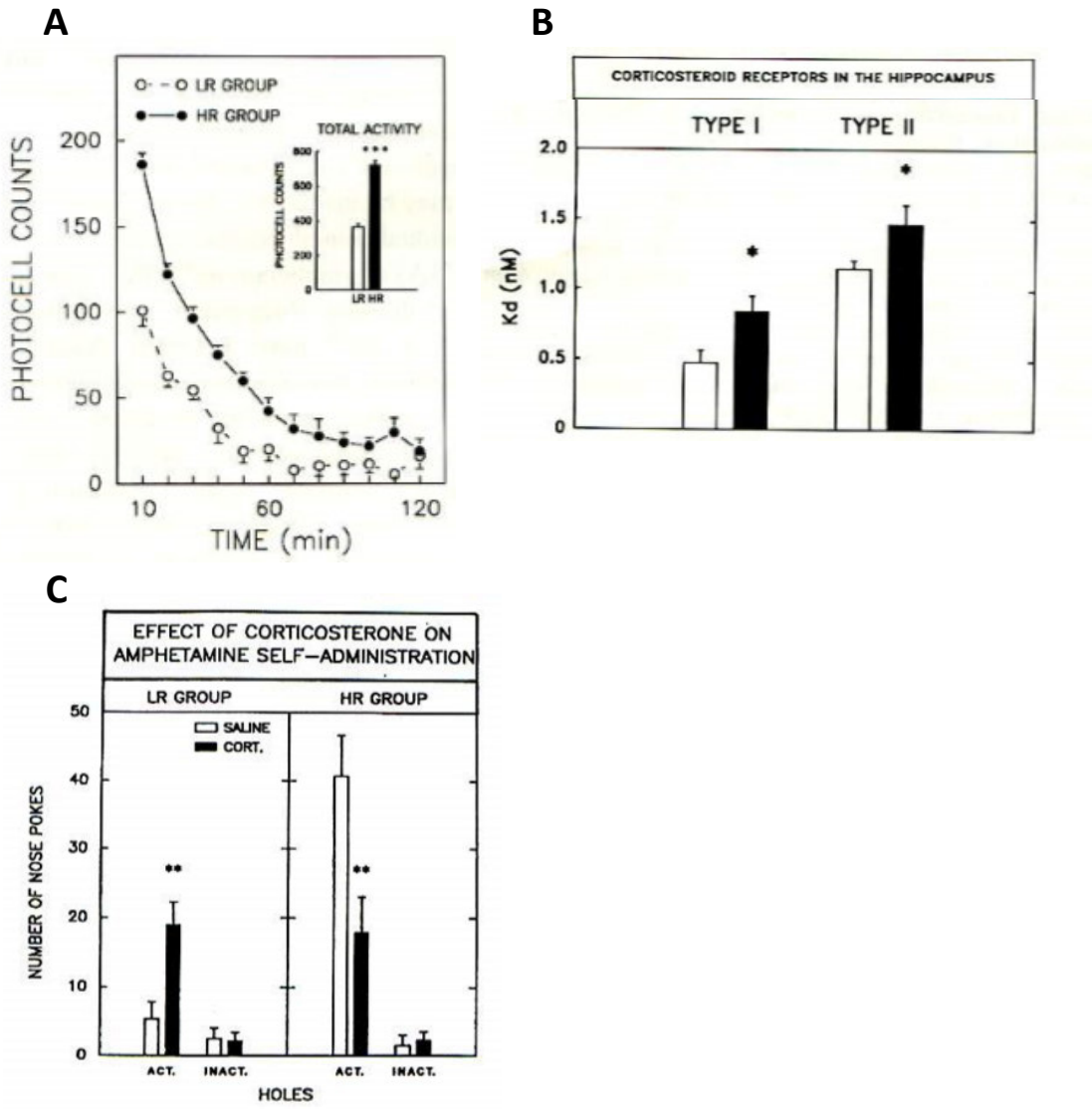


Fig 9- Impact of corticosterone on amphetamine self-administration.

(A) HR rats are characterised by an increased reactivity to novelty, decreased affinities of hippocampal type I and type II receptors and (B) increased amphetamine self-administration respect to LR animals. (C) Intravenous infusion of corticosterone increased the nose pokes in LR animals while it decreased it in HR rats. *P <0.05 vs LR rats and **P <0.05 vs CORT treated group. From Reynaert et al., 2014. Original data are reported in Maccari et al., 1991 (A, B) and Piazza et al., 1991 (C).

From a neurobiological point of view, CRH and other neuropeptides such as dynorphin (Bruchas, Land and Chavkin, 2010) and neuropeptide Y (NPY) (Heilig et al., 1994; Cippitelli et al., 2010) have established roles in linking stress and addiction-related behavior.

Stress is linked to addictive disorders and could promote the emergence of an addiction. In situations of stress, large amounts of glucocorticoids are secreted. But, these hormones increase the sensitivity of the brain to psychotropic and promote the emergence of addictive behaviors in chronically stressed animals (Piazza and Le Moal., 1996, 1998; Marinelli and

Piazza, 2002). Meanwhile, among rats rendered dependent on a substance, administration of molecules that reduce the action of stress hormones has the effect of reducing the consumption in rodents (Koenig and Olive, 2004; Specio et al., 2008).

Glucocorticoid secretion is more or less high depending on the individual, and the hormone concentration determines the susceptibility to addiction (Piazza and Le Moal, 1996). The reverse has also been verified. Indeed, people addicted to cocaine exhibit a heightened sensitivity to stressful events. Stress thus becomes a risk factor of great importance in the relapse phenomenon. Scientific evidence exists in favor of convergence in mechanisms of drug action and stress induction with similar changes in the mesolimbic dopaminergic system (Piazza and Le Moal, 1998) and the central role of glucocorticoids receptor GR in mediating these effects (De Jong and De Klöet, 2004). Recently, a team of French researchers has identified neurons involved in the modulation of stress addiction: these are neurons sensitive to both glucocorticoids and dopamine: selective GR gene ablation in mouse dopaminoceptive neurons expressing DA receptor 1a, but not in DA-releasing neurons, markedly decreased the motivation of mice to self-administer cocaine (Ambroggi et al., 2009).

A glucocorticoids responsive element (GRE), present in the promoter of the tyrosine hydroxylase gene demonstrates the direct interaction between the stress response system and the synthesis of neurotransmitters involved in the response to reward (Hagerty et al., 2001).Of note, adrenalectomy, a surgery that effectively removed the final step in HPA axis activation, the synthesis and secretion of corticosterone inhibits cocaine self-administration (Goeders and Guerin, 1996). These data suggest that plasma corticosterone may be critical for the acquisition of cocaine self-administration to occur in rats.

From this part, emerges the evidence that stress is a very important factor in addiction, but also in other stress-related disorders, like anxiety and depression.

III. INTERINDIVIDUAL VULNERABILITY: SEX DIFFERENCES

Women are significantly more likely to suffer from affective disorders than men, with high prevalence of stress-related disorders such as anxiety and depression; female gonadal steroids are thought to play an important role in the sex difference observed in the incidence of such disorders (Kessler et al., 2005; reviewed by Solomon and Herman, 2009). The same vulnerability to develop anxiety-and depression-like behaviors is also described in animal, where the involvement of sex hormones, in particular estradiol, is clearly described in anxio-/depressive-like profiles (Galea, Wide and Barr, 2001; Bowman, Ferguson and Luine, 2002).

It has been proven that, even if they consume less than men, women were more sensible to the activating effect of drugs on locomotion, and once considering rate of escalation of drug use, women tend to increase rate of drugs consumption more rapidly than do men. Furthermore, once addicted to a drug, women find it more difficult to stop consumption than men do, and, after an abstinence period, women are more vulnerable to relapse. This is true for most drugs of abuse (Becker and Hu, 2008; Fox and Sinha, 2009). An important effect of cycle has been demonstrated in shaping sensitiveness to drugs, and propensity to reinstatement of drug seeking (reviewed by Hudson and Stamp, 2011).

In animal models, a high prevalence of sensitiveness to drugs of abuse has also been demonstrated. For instance, accordingly with the results described by Hu et al. (2004), Lynch (2008) has shown that acquisition of cocaine self-administration was greater in female than in males, and that the percentage of infusions of the drug was also higher in females. Under a progressive ration schedule, the breaking point, reflecting the motivation of the animal to catch the drug, was very high in females in comparison to males. A positive correlation was also found between estradiol concentrations and the number of cocaine infusions. Moreover, the average number of infusions obtained under the progressive-ratio schedule varied with estrous cycle phase with female rats reaching the highest final ratios during estrus as compared to the other phases of their cycle (metestrus/diestrus), consistently with other works finding more important drug craving and reinstatement of drug seeking behavior in females during the estrus phase of the follicular cycle (Kippin et al., 2005). Female rats also exhibit higher response to conditioned place preference for cocaine and require less pairing sessions to develop CPP for cocaine, and with lower doses of drug than males (Russo et al., 2003). Of note, progesterone concurrent administration with estradiol counteracts the estradiol-induced enhancement of cocaine self-administration (Jackson, Robinson and Becker, 2006).

IV. COMORBIDITY BETWEEN ADDICTION AND OTHER STRESS-RELATED DISORDERS

A high prevalence of substance use disorders is observed in patients with major depressive disorders (Davis et al., 2005) or other psychiatric conditions (Kessler et al., 2005) and most patients admitted for drug addiction disorders have a concomitant use of antidepressant (ATD) treatment (Torrens et al., 2005). The high degree of comorbidity between depression and drug dependence indicates that the rates of depression among drug abusers and the rates of drug abuse among depressed patients are substantially higher than expected from the individual rates of these disorders (for review, Markou, Kosten and Koob, 1998). As aforementioned, women are more sensible to the effects of drugs and are more vulnerable to relapse. This would be due to an important comorbidity between psychiatric, mood, anxiety or depression disorders and addiction in women (reviewed by Zilbermann et al., 2003), and an important sensitiveness to the symptoms associated to withdrawal induces a high propensity to relapse in women/females (Ambrose-Lanci, Sterling and Van Bockstaele, 2008; Bock, Goldstein and Marcus, 1996, Back et al., 2005).

It is supposed that the anhedonic symptoms of depression, which constitute the core feature of this illness, would be due to a dysfunctional brain reward system. Thus, alterations in reward and motivational processes at both the behavioral and neurobiological levels may constitute the defining characteristics of both depression and drug dependence. Nevertheless, it is not clear if drug dependence and depression are different behavioral expressions of the same neurobiological abnormalities, or whether one psychiatric disorder leads the other (Markou, Kosten and Koob, 1998).

Khantzian (1985) was to first notice the comorbidity of stress disorders and drug addiction and coined the self medication hypothesis. The self-medication hypothesis invoked to explain why individuals abuse drugs essentially states that the specific psychotropic effects of drugs interact with psychiatric disturbances and painful affect states to make them compelling in susceptible individuals (Khantzian, 1985). This hypothesis thus takes into account the possibility that drug dependence develops as a self-medication in reaction to depression syndrome (Markou, Kosten and Koob, 1998). The idea is that people suffering from addictive disorders use psychostimulants as well as opiates to self-medicate for anxiety or depression (Rounsaville et al., 1982), to improve fatigue, to increase feelings of assertiveness, self-esteem and frustration tolerance. So, many risk factors can predispose an individual to initiate and maintain drug use, such as preexisting mood disorders. Thus, drug abusers are attempting

to medicate themselves for a range of psychiatric disorders and painful emotional states; and it is possible that through the simultaneous use of multiple drugs, people determine the drug or drugs combination that best normalise their behavior. People who experience major trauma in their life may self-medicate with drugs or alcohol to relieve the symptoms of post-traumatic stress disorders and depression (Coffey et al., 2002).

For these individuals, the need to control their depressive symptomatology through self-medication would play an important role in the maintenance of drug dependence. For example, several works indicate that acute administration of psychostimulants such as opioids or amphetamine can temporarily reverse potential neurochemical deficits that may be found in depressed individuals (Tremblay et al., 2002; for review, Markou, Kosten and Koob, 1998). However, none of these drugs of abuse are considered clinically effective as antidepressants by clinicians (Naranjo, Tremblay and Busto, 2001). In any case, the possibility remains that simultaneous or sequential use of various drugs as self-prescribed by the emotional needs of the drug-using individual, leads to an adequate ATD effect, but at the same time pushes the individual in an active state of drug-dependence. The best clinical support for the self-medication hypothesis is provided by the evidence that ATD treatment is significantly more effective in reducing drug use in depressed drug abusers than in non-depressed abusers (Ziedonis and Kosten, 1991a,b; Nunes et al., 1993; 1995). Fluoxetine treatment appears thus efficient in reducing drinking in alcoholic patients with a comorbid history of depression. Some psychoanalysts see addiction as a “protection” against several psychopathological states (depression, psychosis, severe neurosis) (Véléa, 2005). Independently of whether the depression was present before the drug abuse or whether it was drug-induced, the reduction of drug use observed with ATDs suggest that, when there is alleviation of depressive symptomatology through the use of ATD compounds, the need for self-medication with drugs of abuse diminishes (Markou, Kosten and Koob, 1998). Thus, there are several aspects suggesting that these two psychiatric disorders may be linked by some shared neurobiology as well as common epigenetic mechanisms (Nestler et al., 2002; Renthal and Nestler, 2009).

Behaviorally, cocaine use in humans has been reported to produce profound subjective feelings of well being and a decrease in anxiety (Gawin and Ellinwood, 1988, 1989). In fact, a subpopulation of chronic cocaine users may actually be self-medicating to regulate “painful feelings” and psychiatric symptoms *via* their drug use (Kleber and Gawin, 1984; Khantzian, 1985; Gawin, 1986), especially since increased rates of affective disorders and anxiety are observed in these individuals (Rounsaville et al., 1991; Brady and Lydiard, 1992; Kilbey,

Breslau and Andreski, 1992). However, cocaine use, itself, has actually been reported to precipitate episodes of panic attack in some individuals (Anthony, Tien and Petronis, 1989; Aronson and Craig, 1986). Since panic disorder only became apparent following chronic cocaine use in many of these cases, the drug may have functioned as a precipitating as well as a causative factor in neurobiologically vulnerable individuals (Aronson and Craig, 1986). Furthermore, some of the major symptoms observed during withdrawal from chronic cocaine intoxication can often include severe anxiety as well as restlessness, agitation and depression (Gawin and Ellinwood, 1989). Interestingly, CRH has been reported to be involved in a variety of neuropsychiatric disorders including depression and anxiety (Gold and Chrousos, 2013; Gold et al., 1984; Nemeroff, 1988), suggesting that the anxiety associated with cocaine use and withdrawal may depend, in part, on the effects of the drug on the release of this endogenous "stress peptide" and the resulting activation of the HPA axis. A return to drug use can be precipitated by three distinct types of stimuli: (1) exposure to an environmental stimulus (i.e., a discrete or contextual cue) that is strongly associated with the drug experience (Meil and See, 1997; McFarland and Ettenberg, 1997), (2) exposure to a pharmacological stimulus (i.e. the drug itself or a pharmacologically related agent) that induces some component of the drug experience (De Wit and Stewart, 1981; De Vries et al., 1998), and (3) exposure to some stressors (Ahmed and Koob, 1997; Shaham et al., 1997).

We were previously debating on the addictive power of food; it's interesting to see how food can influence mood. In adult rats, the effect of "cafeteria diet" has been assessed on anxiety-like behavior. It has been shown that this kind of diet was able to modify the anxious-like profile of rats. A cafeteria diet decreases the response of male rats to chronic variable stress (Zeeni et al., 2013). This would suggest a potential anxiolytic effect and validate the hypothesis of a stressor-induced preference for high palatable food. An anxiolytic effect in females was expressed by the increased time spent on the aversive open arms. The effects in adult males were increased grooming and fewer entries into the aversive open arms of the EPM apparatus, consistently. In addition, the decreased grooming in females would be in line with an anxiolytic profile in adult females (Warneke et al., 2013). Grooming is a self-directed behavior. When shown upon exposure to aversive situations, as for example the plus maze or the open field, grooming can be interpreted as a de-arousing activity. Anxious rats groom more often and anxiolytic drugs reduce grooming behavior (Dunn et al., 1981; Voigt et al., 2005).

V. PRENATAL STRESS: A FUNDAMENTAL MODEL FOR STUDY OF STRESS-RELATED DISORDERS

1. Prenatal stress: an example of early programming of long term behavioral alterations

In humans, maternal gestational stress (also referred to as prenatal stress) is linked to a variety of negative outcomes for the offspring over development and later in life, such as increased risk for cognitive, emotional, and health-related disorders (Rice, Jones and Thapar, 2007; Talge, Neal and Glover, 2007; Spauwen et al., 2004; Bale et al., 2010). In a retrospective epidemiological study in 1978, Huttunen and Niskanen have demonstrated the impact of maternal stress during pregnancy in psychiatric and behavior disorders. Persons whose fathers had died before their children's births or during the first year of their children's lives showed a higher propensity to suffer from schizophrenia and to commit crimes. Animal models across a wide variety of species have been used to investigate the consequences of stressors in pregnancy and the underlying mechanisms (e.g., guinea pigs (Kapoor and Matthews, 2005); rhesus monkeys (Coe et al., 2003); mice (Son et al., 2006)).

Maternal gestational stress has been investigated most extensively in the rat, with a research history of over 50 years indicating long-lasting physical and behavioral consequences for the offspring (Hartel and Hartel, 1960; Hockman, 1961; Joffe, 1965; Wilson, 1954). Chronic unpredictable stress has thus been shown to induce long-lasting physical and behavioral changes in rats (Cabrera et al., 1999). Stress endured during pregnancy leads to an increase in stress hormones glucocorticoids that can alter placental development and lead to long-term alterations of the offspring (Maccari et al., 1995; 2003; Weinstock, 2005; 2008).

In the present study, we have adopted the prenatal restraint stress procedure, as described in the following part (Fig. 10).

2. The model of Prenatal Restraint Stress (PRS) in rat

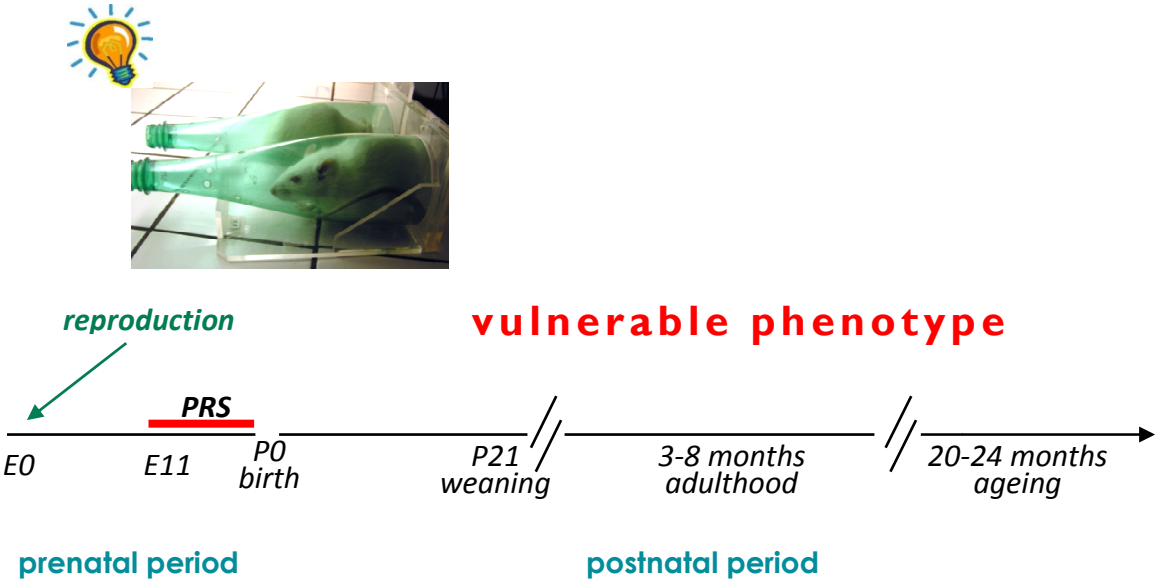


Fig 10- The Prenatal Restraint Stress (PRS) model in rat.

The procedure of prenatal restraint stress is conducted as previously described (Maccari et al., 1995; Morley-Fletcher et al., 2003). Male and female Sprague Dawley rats, provided by Charles River Laboratories are placed in the animal facility for 15 days for stabilization, under controlled temperature and hygrometry. Females are placed 10 per cages for cycle synchronization while males stay alone. Then, two females are placed with a male for reproduction for overnight. The next day, the vagina smear is observed by microscopy. The visualization of spermatozoids or of plug indicates the beginning of gestation and is determined as E0 (Embryonic day 0). On E11, some females are submitted to restraint stress, 3 times per day for 45 min under bright light. Control unstressed females are left undisturbed, exception made for weighing one time per week to follow gestation. On weaning (Postnatal day (P21), male and female rats are separated and sisters/brothers are placed by 2-3 per cage.

2.A. HPA axis and maternal factors

Enhanced corticosterone secretion by stressed mothers during pregnancy has been shown to play a key role in the programming of a pathological phenotype in the offspring (Kapoor et al., 2006). Indeed, blocking stress-induced maternal corticosterone secretion by adrenalectomy in stressed dams suppressed stress consequences on the offspring, in particular the HPA axis alterations (Barbazanges et al., 1996) that are long-lasting hallmarks in prenatally stressed rats (Henry et al., 1994). Of note, the administration of corticosterone injections (that reproduced the surge in plasma corticosterone levels in intact stressed mothers) to these dams, concomitantly with restraint stress sessions, was able to reinstate the effects of PRS on the offspring (Barbazanges et al., 1996).

In others prenatal stress models, where stress is applied during late gestation period, and once daily, maternal adrenalectomy was also able to attenuate some of the effects of gestational stress on the onset of behavioral alterations, such as anxiety-like behavior or spatial memory impairments (Zagron and Weinstock, 2006).

Furthermore, in a large number of animal studies, treatment of pregnant dams with the synthetic glucocorticoid dexamethasone, which readily crosses the placenta, has been shown to induce in the offspring hallmarks similar to the ones induced by prenatal stress. Thus, prenatal dexamethasone exposure has been implicated in the development of hyperglycemia and hypertension in the adult, as well as changes in physical and behavioral phenotype (such as low body weight, anxious-like behavior, learning impairments) and alterations in HPA axis function (Shoener, Baig and Page, 2006; Benediktsson et al., 1993; Levitt et al., 1996; Welberg, Seckl and Holmes, 2001; Dean and Matthew, 1999, Dunn et al., 2010). Moreover, exposure of pregnant rats to alcohol (a procedure that stimulates maternal glucocorticoid secretion) results in a hyper-active HPA axis in the offspring (Lee et al., 2000).

Similar associations between low birth-weight and hyperactivity of the HPA axis in adulthood are seen in human cohorts (Phillips et al., 1998; Reynolds and Phillips, 1998; Levitt et al., 2000). Likewise, non-abortive maternal infections, which also increase maternal glucocorticoids (Besedovsky et al., 1975), compromise the development of the fetal brain and alter HPA axis function in the adult (Reul et al., 1994). Maternal hormones thus seem to be good candidates for communication between the dam and the developing fetus (Joffe, 1969; 1978).

Furthermore, the activity of l1β-hydroxysteroid dehydrogenase type 2 (ll β-HSD2), an enzyme which catalyzes the rapid inactivation of cortisol and corticosterone to inert 11 keto-products and protects the fetus from excess maternal glucocorticoids (Seckl, 1997; Benediktsson et al., 1997), is decreased in the placenta of prenatally restraint stressed rats, with an ensuing increase in maternal corticosterone reaching the fetus (Mairesse et al., 2007). In both rats and humans, there is considerable natural variation in placental l1β-HSD2, and enzyme activity correlates with birth weight (Seckl, 1997) and has critical effect on adult neurogenesis (Lucassen et al., 2009). Moreover, inhibition of feto-placental 11β-HSD2 in the rat reduces birth weight, produces hypertensive and hyperglycemic adult offspring and leads to permanent alterations of the HPA axis and anxiety-like behavior in aversive situations (Edwards et al., 1993; Welberg, Seckl and Holmes, 2000).

In addition to the excessive secretion of glucocorticoids consequently to stress, other maternal factors could contribute to the long lasting effects of PRS (Darnaudéry and Maccari, 2008). Indeed, the stress procedure has consequences on pregnant dams themselves, with increased anxiety, weaker increase in body weight gain during pregnancy and impaired coping in inescapable situations (Darnaudéry et al., 2004) as well as post-partum depression-like behavior (Smith et al., 2004; O'Mahony et al., 2006). These behavioral troubles have profound impact in the offspring, as maternal prenatal anxiety and stress predict infant illnesses and health disorders (Beijers et al., 2010).

In experiments performed in rats selectively and bidirectionally bred for high (HAB) and low (LAB) anxiety-related behavior, a well-established as a suitable animal model of anxiety- and depression-related disorders (reviewed by Landgraf and Wigger, 2002), a significant increase in ACTH and corticosterone levels in response to a stressor was observed in HAB rats (Neumann, Krômer and Bosch, 2005).

In the PRS model, it has been shown that stress endured during gestation had long–lasting effects with behavioral impairments always seen after weaning whereas chronic restraint stress had no effect on body weight growth (measured 7 days after the end of the stress period), on behavioral parameters and on corticosterone levels after exposure to novelty, determined 3-5 weeks after the end of the stress period (Darnaudéry et al., 2004).

These results suggest that the perinatal period is critical and that it is preferable to refer to the stress as "perinatal" rather than simply "prenatal" (Darnaudéry and Maccari, 2008).

This concept is also supported by the evidence that in the PRS model, gestational stress alters active maternal behavior towards pups (Mairesse, Gatta, Reynaert et al., submitted, *J. Neurosci*), consistently with other works with different maternal stress procedures showing a

decrease in active behavior engaged toward pups in favor to self-directed activities (Baker et al., 2008; Champagne and Meaney, 2006; Smith et al., 2004, Patin et al., 2002). Weaver et al., in 2004, were also able to demonstrate the impact of maternal stress on offspring HPA axis response through epigenetic mechanisms.

Since adoption, postnatal handling or environmental enrichment are able to abolish prenatal stress consequences (Maccari et al., 1995; Vallée et al., 1997; Lemaire et al., 2006; Koo et al., 2003; Morley-Fletcher et al., 2003b; Laviola et al., 2004), the importance of perinatal period in programming a vulnerable phenotype in the offspring is fully confirmed. Of note, neonatal handling enhances maternal care and attenuates specific dexamethasone-induced alterations in the adult behavioral phenotype (Claessens et al., 2012).

Interestingly, oxytocin administration, through its analogue carbetocin, to lactating mothers was able to improve maternal behavior and to abolish the prenatal-stress-induced programming of a pathological phenotype in the offspring, considering both corticosterone secretion in response to a stressful situation and anxiety-like behavior in the elevated plus maze paradigm (Mairesse, Gatta, Reynaert et al., submitted, *J. Neurosci*).

2.B. HPA axis, anxious- and depressive-like behavior

HPA axis

PRS rats, i.e. the progeny of dams submitted to restraint stress during the last 10 of their gestation (Fig. 10) display a long-term impairment of the HPA axis functioning with a prolonged corticosterone secretion in response to stress (Maccari et al., 1995, 2003; Koehl et al., 1999, Vallée et al., 1997) and reduced levels of both mineralocorticoid and glucocorticoid receptors in the hippocampus (Maccari et al., 1995; Henry et al., 1994; Van Waes et al., 2006). The age-related HPA axis dysfunctions are enhanced by PRS. Indeed, the HPA axis period of hyporesponsiveness was reduced in new-born PRS rats with respect to their controls (Henry et al., 1994) and circulating glucocorticoid levels of PRS middle-aged animals were similar to those found in old non-stressed animals (Vallée et al., 1999).

A significant phase advance in the circadian rhythms of corticosterone secretion and locomotor activity was also found in prenatally-stressed (PNS) rats (Koehl et al., 1997).

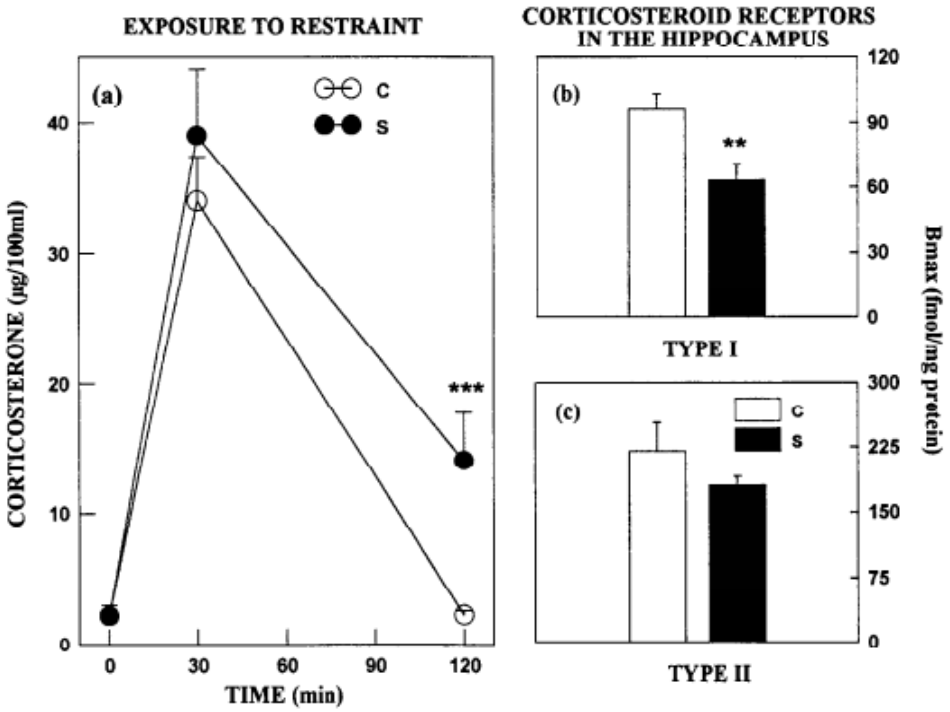


Fig 11- Plasma corticosterone secretion after restraint stress (a) and type I (b) and type II (c) corticosteroids receptors in adult prenatally unstressed (C) and stressed (S) rats.

a, Prenatally stressed animals did not differ from controls for corticosterone levels in basal conditions or after 30 min exposure to restraint stress. However, corticosterone levels remained high in S rats 120 minafter stress, whereas they returned to preexposure values in the controls. b, S rats showed a lower binding capacity (+ 40%) of type I corticosteroids receptors than C. c, Prenatal stress did not significantly modify type II corticosteroids receptors. The affinities of type I and type II receptors were not modified by PRS. From Maccari et al., 1995.

Anxiety

The increased anxious/depression-like behavior is already evidenced in infancy by increased ultrasonic vocalisations in PRS pups (Laloux et al., 2012).

A reduced exploration of the open arms in the elevated-plus maze (EPM) test and of the centre in the open field test was seen in adult PRS male rats (Vallée et al., 1999). Interestingly, a different phenotype was obtained in females, which appeared less anxious than control females, and spent more time in the open arms of an EPM apparatus, demonstrating a clear-cut sex dimorphism for this parameter (Zuena et al., 2008). A recent work of the team has identified glutamate as a key factor in the anxiety-like profile displayed by PRS rats (Marrocco et al., 2012). We have shown that PRS induced a selective reduction of glutamate release in the ventral hippocampus that is causally related with the enhanced anxiety-like behavior displayed by PRS animals, since pharmacological enhancement of glutamate release in the ventral hippocampus abolished this behavioral pattern (Marrocco et

al., 2012). Remarkably, most of the neuroplastic alterations induced by PRS in males occur prominently in the ventral portion of the hippocampus a key region in the regulation of stress, emotions (Kjelstrup et al., 2002; Fanselow and Dong, 2010).

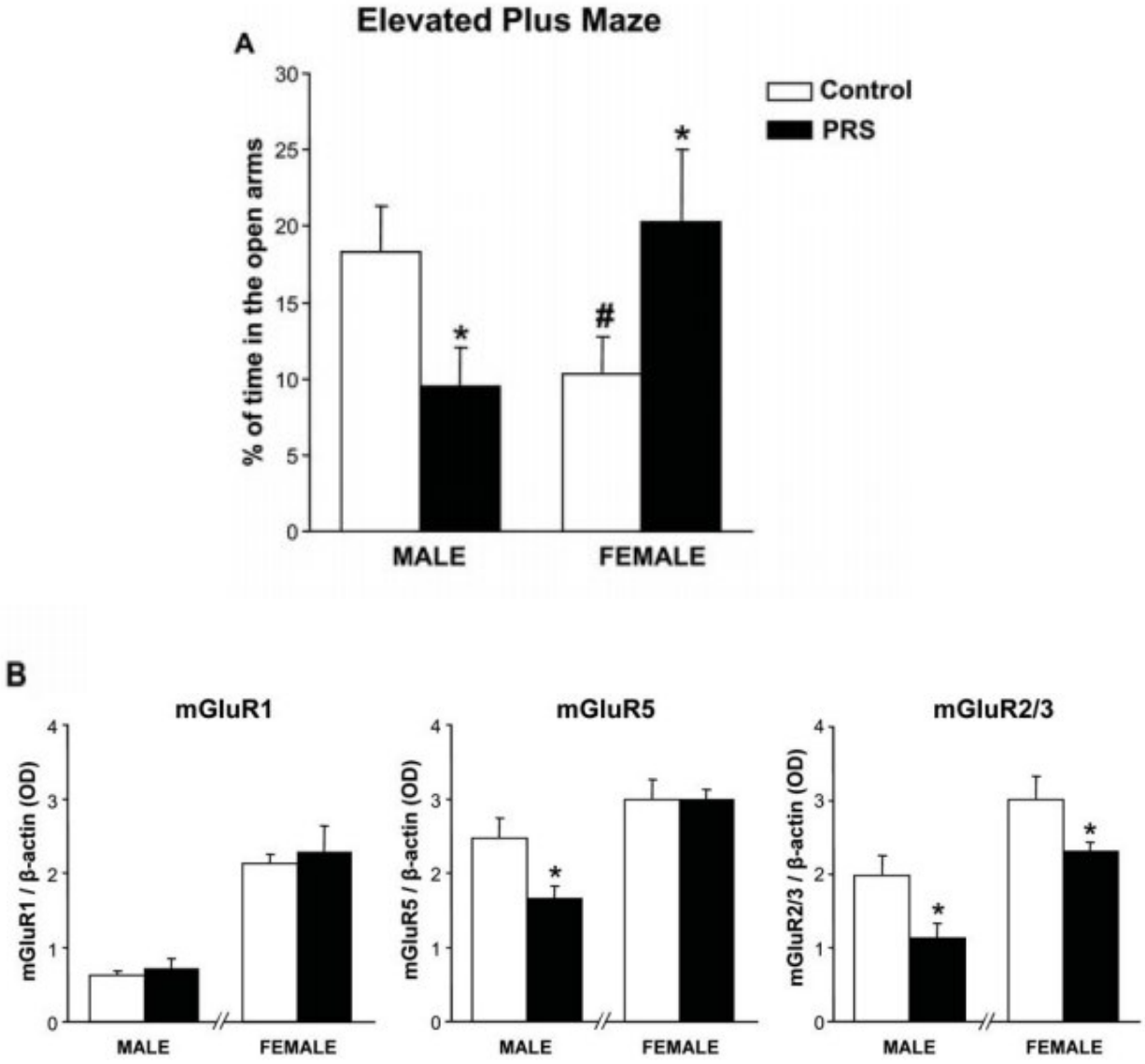


Fig 12- Changes in anxiety-like behavior (A) and expression of group-I and group-II mGlu receptors in the hippocampus (B) induced by PRS in male and female rats.

The time spent by Control and PRS rats in the open arms of the EPM is shown in (A). A clear-cut gender effect of PRS in the EPM is obtained. Immunoblots are shown in (B). In male rats, PRS induced a reduction in the expression of mGlu5 receptors but no change in the expression of mGlu1a receptors. PRS had no effect on the expression of mGlu1a and mGlu5 receptors in female rats. Interestingly, PRS reduced mGluR2/3 receptor expression in the hippocampus of both male and female rats. Adapted from Zuena et al., 2008.

Depression

In PRS rats, an increased immobility in the forced-swim test (FST) has been shown during adulthood (Morley-Fletcher et al., 2003a; 2004a; 2011). In this test, which enables a screening of the effect of antidepressant (ATD) (Porsolt et al., 1978), PRS rats were more prone to develop a depressive-like phenotype; and this vulnerability was also found in female PRS rats (Van Waes et al., 2011), consistently with other works describing a behavioral despair in prenatally stressed females in the FST (Alonso et al., 1991, 1997). Our group has provided increasing evidence for the predictive validity of PRS model by means of chronic treatment with different classes of antidepressants in adult rats. The anxious-/depressive-like phenotype observed in prenatally stressed rats can thus be corrected by chronic treatment with the ATD tianeptine, a selective serotonin reuptake enhancer, structurally similar to tricyclic ATDs (Morley-Fletcher et al., 2003a), with agomelatine, a dual ATD with melatonergic agonist and 5-HT2C antagonist properties (Maccari and Nicoletti, 2011; Morley-Fletcher et al., 2011) or with amitriptyline, imipramine and nomifensine (Alonso et al., 1999). ATDs beneficial effects are observed at the behavioral, neurochemical and neuroanatomical levels.

Thus, following ATD treatment, PRS rats displayed reduced immobility behavior in the FST, increased exploration of the open arm in the elevated-plus maze, enhanced MR and GR densities in the hippocampus and modified 5-HT1A mRNA expression (Morley-Fletcher et al., 2003a and Morley-Fletcher et al., 2004a). Also, since preclinical and clinical research has increasingly focused on the interaction between stress and depression and their effect on hippocampus (Duman, Malberg and Nakagawa, 2001), we have recently tested the effects of antidepressant treatment on hippocampal neurogenesis. Interestingly, PRS induces a life span reduction of hippocampal neurogenesis in male rats (Lemaire et al., 2000; Lemaire et al., 2006) but not in females (Darnaudéry et al., 2006), and postnatal stimulation counteracts prenatal stress-induced deficits in hippocampal neurogenesis (Lemaire et al., 2006). Chronic treatment with agomelatine is also able to increase hippocampal neurogenesis, especially in the ventral part of the hippocampus of male rats (Morley-Fletcher et al., 2011). This latter finding gives further support to the validity of the PRS model.

The effect of prenatal stress on the sleep-wake cycle was also investigated in adult male rats, sleep being another parameter of depression with various clinical observations in humans suggesting a possible pathophysiological link between depression and disturbances in circadian rhythmicity (Rosenwasser and Wirz-Justice, 1997; Holsboer, 2001). Prenatally stressed rats exhibited various changes in sleep-wake parameters, including a dramatic

increase in the amount of paradoxical sleep (Dugovic et al., 1999; Mairesse et al., 2013), with a significant enhancement in REM sleep over a 24-h recording session, positively correlated to plasma corticosterone levels. Added to our previous findings in PRS rats of high anxiety and emotionality, dysfunction of the HPA axis and circadian timing abnormalities, the observation of long-term changes in their sleep structure supports the validity of the PRS model as an animal model of anxiety/depression (Maccari and Morley-Fletcher, 2007).

Again, ATD treatment was able to correct abnormalities in sleep architecture alterations in PRS rats (Mairesse et al., 2013). Those results reinforce the idea of the usefulness of PRS in rats as an appropriate animal model to study human depression and support the use of new ATDs.

2.C. Drug addiction

Several studies have revealed that prenatal restraint stress enhanced sensitiveness to drugs of abuse. Enhanced psychomotor activation in response to stimulants occurs in PRS male rats PRS rats challenged with amphetamine (Deminière et al., 1992), cocaine (Kippin et al., 2008) or nicotine (Koehl et al., 2000; Fig. 13), and PRS rats exhibit increased drug seeking behavior (Deminière et al., 1992; amphetamine; Fig. 14; Thomas et al., 2009, cocaine) and greater behavioral sensitization to the drug (Henry et al., 1995). After repeated injections of cocaine, prenatally stressed female but not male rats displayed a greater locomotor response after repeated injections of cocaine (Thomas et al., 2009).

PRS also enhances sensitivity to MDMA “ecstasy” motor alterations and MDMA pharmacokinetics in adolescent female rats (30 days) (Morley-Fletcher et al., 2004b). We found that PRS increased levels of plasmatic MDMA during the kinetic assessment with respect to controls and induced a higher frequency of altered motor co-ordination following MDMA administration, thus indicating a strong consistency between drug blood levels and behavior.

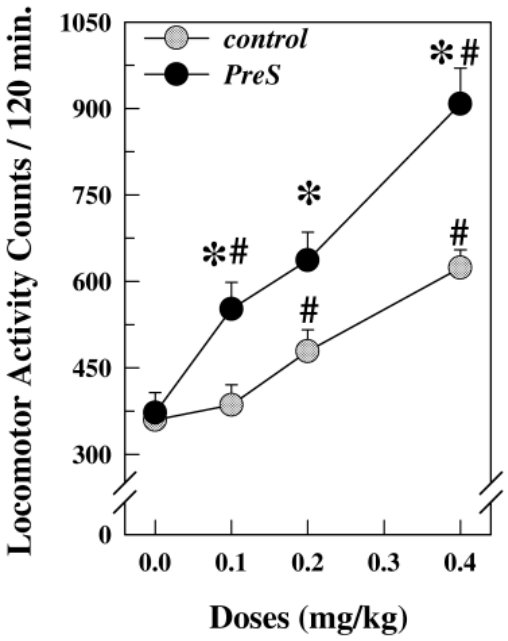


Fig 13- Effects of acute injections of different doses of nicotine in control and prenatally-stressed (PreS) rats. Nicotine induces a dose-dependent increase in locomotor activity in both groups (#, different from the previous dose, $p<0.05$). Furthermore, the locomotor response to each dose of the drug tested is enhanced in the prenatally stressed group (*, different from control animals, $p<0.05$). From Koehl et al., 2000; Reynaert et al., 2014

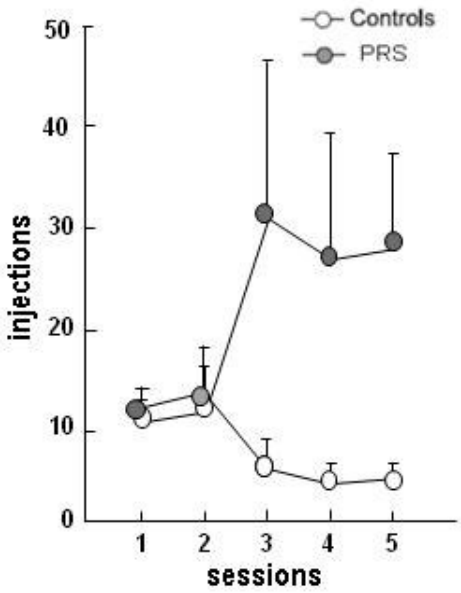


Fig 14- Self administration of amphetamine in Control and PRS rats.

PRS rats display a significant increase in amphetamine self-administration. Adapted from Deminière et al., 1992; Reynaert et al., 2014.

In addition to psychostimulants, PRS also modulates response to alcohol. Several studies have been conducted on the effect of chronic ethanol treatment on PRS rats and measured the effects of alcohol exposure in both male and female PRS rats during adolescence, adulthood or ageing. Male adolescent PRS rats show a reduced activation of the HPA axis in response to acute alcohol administration (Van Waes et al., 2006), a phenomenon observed in heavy drinkers and their relatives, as well as in alcohol-dependent rats.
It has also been clearly demonstrated that prenatal dexamethasone exposure is a potent factor that could directly influence the development of central monoaminergic systems such as the noradrenergic, dopaminergic, and serotonergic systems (Muneoka et al., 1997). Taken together, these results suggest that the disruption of the normal hormonal response to stress observed in PRS individuals and developmental alterations in brain monoamine metabolism depend, at least in part, on stress-induced increases in maternal glucocorticoids during pregnancy (Darnaudéry and Maccari, 2008).

An extensive literature has emerged examining the neural circuits and cellular mechanisms through which stress modulates addictive behavior (Koob, 2008). Sensitization of the dopaminergic response to drugs is considered the neural substrate of behavioral sensitization and has been implicated in vulnerability to drug abuse. The HPA axis plays a key role in the development of sensitization to psychostimulants. This idea is further supported by the presence of glucocorticoids receptors in the dopaminergic neurons of the ventral tegmental area (VTA) projecting in the NAc (Härfstrand et al., 1986). Glucocorticoids promote sensitization to psychostimulants in rats (Rivet et al., 1989; Maccari et al., 1991; Piazza et al., 1991; Deroche et al., 1995), while administration of psychostimulants has been shown to activate the HPA axis (Swerdlow et al., 1993). Glucocorticoids control stress-induced sensitization by changing the sensitivity of the mesencephalic dopaminergic transmission to drugs of abuse. There is also evidence for control of corticosteroid receptors by dopamine, since dopamine inhibits the expression of corticosteroid receptors in the anterior pituitary (Antakly, Mercille and Côté, 1987; Casolini et al., 1993), while administration of amphetamine decreased the concentration of corticosteroid receptors (Lowy, 1990). Several studies conducted on PRS rats have evidenced functional alterations in mesolimbic dopamine system. In particular, PRS induces a significant increase in DRD2 receptor binding and mRNA in the core region of the NAc (Henry et al., 1995; Berger et al., 2002; Rodrigues et al., 2011), and a marked decrease in DRD3 receptor binding in both the shell and the core of the NAc (Henry et al., 1995).

Moreover, basal and amphetamine –stimulated DA output in adolescent and adult PRS rats is higher than in control unstressed rats when measured by microdialysis in the shell region of the NAc (Silvagni et al., 2008). Recently, Hauskenecht, Haj-Dahmane and Shen (2013) have shown that a PRS paradigm that leads to increased locomotor activity to novelty and enhanced response to amphetamine also causes a persistent reduction in the spontaneous activity of VTA DA neurons recorded in adult animals. Such reduction of neural activity can be reversed by acute apomorphine that normally inhibits the impulse activity of DA neurons. Furthermore, the reduced number of spontaneously active VTA DA neurons can be also reversed by acute psychostimulants (i.e. amphetamine; cocaine), which in control rats inhibited the activity of VTA DA neurons. These findings lead to suppose that the reversal effect on VTA DA neurons observed in PRS animals represents an actual increase in the impulse activity. This effect could contribute to the increased responding to psychostimulants and mediate the increased addiction risk after prenatal stress (Hauskenecht, Haj-Dahmane and Shen, 2013).

The administration of drugs of abuse, including cocaine and nicotine, also increases glutamatergic neurotransmission in brain structures implicated in the regulation of reward processes, such as the dorsal striatum (McKee and Meshul 2005), NAc (Pierce et al., 1996) and VTA (Kalivas and Duffy, 1998; Schilstrom et al., 2000). Moreover, drug-induced adaptations in glutamatergic neurotransmission have been suggested to be involved in the development of drug dependence (Nicoletti et al., 2011; Battaglia et al., 2002; Kalivas, Volkow and Seamans, 2005; Liechti and Markou, 2008). Interestingly, Kippin and coworkers (2008) have shown that PRS rats have a heightened corticolimbic dopamine and glutamate response to cocaine. Indeed, cocaine-naıve PRS rats exhibited increased NAc dopamine and reduced NAc serotonin and glutamate, while cocaine-experienced PRS rats exhibited enhanced NAc glutamate and dopamine and PFC dopamine neurotransmission. Recently, we have increasing evidence of an involvement of the glutamate transmission and glutamate machinery in particular at the level of mGlu receptors in the neuroplastic programming and behavioral phenotype induced by PRS (Marrocco et al., 2012). PRS rats also show a reduced expression and function of group-I and group-II mGlu hippocampal receptors, with a marked reduction of mGlu1 and mglu5 selectively for males and mglu2/3 for both males and females (Zuena et al., 2008; Van Waes et al., 2009; Laloux et al., 2012). The alterations in mGlu receptors induced by PRS in the hippocampus are detectable already at infancy with mGlu5 and mGlu1 receptors reduced in infant PRS rats at postnatal day 10, whereas expression of mGlu2/3 receptors declined only after weaning (Laloux et al., 2012). Metabotropic glutamate

receptors are clearly involved in the reinforcing and hyperlocomotor effects of several drugs of abuse. Mice lacking mGlu5 receptor do not self- administer cocaine or display cocaine-induced hyperlocomotion (Chiamulera et al., 2001). Antagonists for mGlu5 receptor reduce drug self-administration for cocaine (Kenny et al., 2003; 2005), nicotine (Paterson and Markou, 2005) and ethanol (Backstrom et al., 2004). Localization studies have indicated a high abundance of mGlu5 receptors in brain areas involved in reward processes, including the striatum and NAc (Testa et al., 1994), further supporting the involvement of mGlu5 receptors in brain reward function.

Chronic treatment with ATDs correct mGlu5 and mGlu2/3 expressions in the hippocampus of PRS male rats (Morley-Fletcher et al., 2011) and, ethanol treatment modulates hippocampal mGlu1a expression and related behavioral changes in both male and female PRS rats (Van Waes et al., 2009; 2011). In particular, chronic ethanol treatment had no effect on hippocampal mGlu5 or mGlu2/3 expressions in PRS males while it increased mGlu1a receptor levels and had an effect on improving memory. On the other hand, chronic ethanol reduced mGlu1a and induced memory impairment in control unstressed male rats (Van Waes et al., 2009). In females, chronic exposure to ethanol during adolescence reduced mGlu1a receptor levels in PRS females while it increased it in control unstressed females. Those effects were still evident after 5-weeks of ethanol withdrawal (Van Waes et al., 2011), whereas control rats also displayed depressive-like behavior. Since mGlu1 receptor antagonists show antidepressant-like effects in the forced-swim test (Belozertseva et al., 2007), the increase in mGlu1a receptor levels we have seen in the hippocampus of unstressed female rats after five weeks of ethanol withdrawal is in agreement with the depressive-like behavior in the forced swim test. Taken collectively, these data suggest a potential use of mGlu1 receptor antagonists in the treatment of depressive symptoms associated with alcoholism. This expands the possible applications of mGlu1 receptor antagonists in human disorders. It remains to be determined whether changes in mGlu receptors in PRS rats following psychostimulants administration are also observed for mGlu5 and mGlu2/3 subtypes in the hippocampus and in reward-related brain regions.

Table 1 – Summary of the main effects of prenatal restraint stress on the HPA axis, metabolic and immune functions and behavior in male and female offspring

HPA axis dysfunctions	Metabolic, immune dysfunctions	Behavioral dysfunctions
Hippocampal MR and GR receptors: ⬇ of mRNA in the hippocampus of adolescent males: main effect in the CA3 (Van Waes et al., 2006); ⬇ maximal binding capacity in adult males (Henry et al., 1994; Maccari et al., 1995; Koehl et al., 1999) and females (Koehl et al., 1999). **Adrenal gland weight:** ⬇ at birth in males (Lesage et al., 2004; Mairesse et al., 2007a); ⬆ in adolescent, adult, and 10 month-old males (Lemaire et al., 2000) and in 26 month-old females (Darnaudery et al., 2006). **Corticosterone secretion:** *Basal levels*: ⬇ at birth in males (Lesage et al., 2004); ⬆ at the end of the light phase in males and throughout the cycle in females (Koehl et al., 1997, 1999); ⬆ in old males (Vallée et al., 1999). *After novelty stress (corridor circular, restraint, elevated plus-maze)*: suppression of the stress hyporesponsive period in infant rats (Henry et al., 1994); ⬇ of the sensitivity of the negative feedback of the HPA axis in adolescent, adult and 16 month-old males (Henry et al., 1994; Maccari et al., 1995; Barbazanges et al., 1996; Vallée et al., 1997, 1999; Morley-Fletcher et al., 2003a,b; Viltart et al., 2006). ⬆ of the corticosterone response in 26 month-old females (Darnaudery et al., 2006). *After pharmacological stress (alcohol)*: ⬇ of the HPA response (CRH mRNA, POMC mRNA, corticosterone levels, ACTH levels) after an acute exposure to alcohol (1.5 g/kg, i.p.) in adolescent males (Van Waes et al., 2006).	**Body weight:** ⬇ at embryonic day 21 in male and female fetuses (Lesage et al., 2004; Mairesse et al., 2007a,b); ⬇ in adolescent males (Van Waes et al., 2006); ⬇ in adult males (Vallée et al., 1996). **Glycemia:** ⬇ at embryonic day 21 in male fetuses (Lesage et al., 2004); ⬆ in 5 month-old adult males (Vallée et al., 1996) and in 24 month-old males (Lesage et al., 2004). ⬆ after an oral glucose tolerance test in 24 month-old males (Lesage et al., 2004). **Feeding behavior:** ⬇ in adult males (Vallée et al., 1996). ⬆ more important after a fasting episode in 24 month-old males (Lesage et al., 2004). **Glucose transporter proteins in the placenta:** ⬆ GLUT 1, ⬇ GLUT 3, GLUT 4 (Mairesse et al., 2007a). **Immune function:** ⬇ $CD4^+$ cells, ⬆ IL-1β levels in splenocytes and in brain, in 34–35 day-old adolescent males (Laviola et al., 2004). In 6 month-old males: ⬆ $CD8^+$; ⬆ NK cells. ⬆ proliferation of T lymphocytes, ⬆ secretion of IFN-γ after stimulation in vitro by phytohemagglutinin-A (Vanbesien-Mailliot et al., 2007).	**Anxiety — depression:** ⬇ exploration in the open arms of the elevated plus-maze (Vallée et al., 1997); ⬆ reactivity to novelty in adult males (Deminiere et al., 1992; Vallée et al., 1997) and in adult females (Louvart et al., 2005). ⬆ number of paradoxal sleep episodes in 3 month-old males (Dugovic et al., 1999). ⬆ immobility in the forced-swim test; ⬇ immobility after chronic antidepressant treatment in adult males (Morley-Fletcher, 2003a, 2004a). **Drug of abuse:** ⬆ amphetamine self-administration in adult males (Deminiere et al., 1992); ⬆ resistance to extinction to cocaine self-administration and ⬆ cocaine-primed reinstatement (Kippin et al., 2007). ⬆ locomotor response to amphetamine (Deminiere et al., 1992; Henry et al., 1995) and nicotine (Koehl et al., 2000) in adult males. ⬆ motor impairments after MDMA (Ecstasy) in 30 day-old adolescent females (Morley-Fletcher et al., 2004b). Maintained high consumption levels after footshock in high-preferring adult females (Darnaudery et al., 2007). **Learning and memory:** ⬇ spatial learning performances in 1 month-old and 3 month-old females (Wu et al., 2007) and in 4 month-old adult males (Lemaire et al., 2000) in the water maze (reference memory). ⬇ spatial learning performances in the water maze (reference memory) in 26 month-old females (Darnaudery et al., 2006). ⬇ spontaneous spatial recognition in juvenile (4 weeks) males and females (Gue et al., 2004) and in 15 and 21 month-old male (Vallée et al., 1999); ⬇ working memory performances in a radial maze in 22 month-old males (Vallée et al., 1999).

Fig 15- Alterations induced by Prenatal Stress.

From Darnaudéry and Maccari, 2008.

We have shown that the pathological epigenetic programming triggered by early life stress predispose to drug abuse disorders and anxiety/depression like behavior (for review, Maccari and Morley-Fletcher, 2007; Darnaudéry and Maccari 2008). Thus, the known co-morbidity between anxiety/depressive disorders and addiction should be interpreted within a context that takes into account the complex interaction between early life experiences and stressful event occurring during adolescence and or adulthood.

As example, we have shown that ethanol intake during adolescence induced depression-like effects in the adult life. Remarkably, the reverse was also true, i.e. ethanol intake during adolescence prevented the "depressive behavior" otherwise seen in adult PRS rats (Van Waes et al., 2011). Perhaps, the increase in ethanol preference observed in adolescent PRS female rats might reflect a strategy of self-medication aimed at preventing the onset of depressive disorders later in life.

Therefore, the PRS model in the rat should be considered as a good model for the investigation of *multiple inter-related pathologies*. Since its effects are persistent through life span, it represents also a more advantageous animal model with respect to other models that present the same pattern of coexistence of these diseases (chronic mild stress), but have transitory effects (effects observable up to 2 months after termination of stress procedure).

As a whole, the existence of comorbidity in depression and drug abuse, underlines the importance of the adoption of an integrated approach in the treatment of these disorders, where the brain reward system could be considered also as a potentially important therapeutic target. An elucidation of the neurobiological and behavioral mechanisms mediating this comorbidity would not only lead to the development of better treatments for these two psychiatric disorders, but would also enhance our understanding of the mechanisms sub-serving motivational and affective processes in both healthy and diseased individuals. Finally, considering that all psychiatric disorders including depression and drug dependence, involve primarily behavioral symptoms that reflect underlying neurobiological abnormalities, progress in understanding these diseases at any level of analysis would certainly involve a multidisciplinary research approach.

In this context, it would be interesting to explore hypothesis generated in the field of depression in animal models of drug dependence and vice versa. Furthermore, exploration of the self-medication hypothesis should be aimed at testing whether various drugs of abuse reverse depressive symptomatology in animal models of depression.

AIM OF THE THESIS

As described in the introduction, several factors intervene in the individual propensity to drug addiction. Both in humans and animal models, stress, sex, as well as comorbidity with mood disorders, appear as key factors in determining the vulnerability to drug addiction (Keyes, Hatzenbuehler and Hasin, 2011). In animal models of early stress, it has been shown that stress-related events that occur during the fetal and early postnatal period may have lifelong programming effects on the HPA axis functioning and different body functions, with a considerable impact on disease susceptibility (Maccari et al., 1995; Darnaudéry and Maccari, 2008; Weinstock, 2005; 2008). The use of animal models involving early-life environmental manipulations allows the study of individual vulnerability applied to stress-related disorders and contributes to improve drug abuse treatment by developing new therapeutic strategies.

The PRS model in rat is characterized by high propensity to drug abuse (Déminière et al., 1992; Morley-Fletcher et al., 2003; Koehl et al., 2000; Kippin et al., 2000), concomitant with an alteration of the HPA axis (Maccari et al., 1995) and an anxious-/depressive-like phenotype (Zuena et al., 2008; Marrocco et al., 2014). Sex differences exist in prenatal epigenetic programming (Bale, 2011). A clear-cut sex dimorphism has been shown in relation to anxiety (Zuena et al., 2008), with PRS male but not female rats showing anxiety-like behavior while, in contrast, both male and female PRS rats display depressive-like phenotype (Morley-Fletcher et al., 2011; Van Waes et al., 2006).

Early life events are important in shaping the neurobehavioral adaptations to environmental challenges in both sexes, and support the emerging consensus on early origins of drug vulnerability and depression and as potentially interdependent stress-related disorders (Maccari and Morley-Fletcher, 2007). Although studies have investigated the impact of PRS in the programming of an anxious-/depressive-like phenotype and addictive disorders, nothing is known concerning a putative causal link between these disorders and the real role of sex and sex hormones in the modulation of the neurobehavioral phenotypes.

Within this context, the objective of my thesis was to bring further elements in the **characterization of both anxious-/depressive-like behavior and addictive-like disorders in the PRS rat model**.

I chose **to assess addiction behavior** in the **Conditioned Place Preference paradigm** (CPP) since it is an experimental protocol that allows measurements of motivational behavior in

conformity with the "natural behavior" of the animal. I also evaluated the **role of gender, sex hormones in particular, in modulating PRS phenotypes** in male and female rats.
Then, I examined how the greatest propensity to addiction observed in PRS rats could be explained by an anxiolytic/antidepressive effect of drugs of abuse, in a context of **self-medication strategy**.

In Chapter 1, we wanted to further investigate the role of glutamatergic transmission in the predictive validity of the PRS model as a model of anxiety/depression and the impact of gender and sex hormones in shaping the vulnerability to develop these disorders.

a) We assessed the effect of chronic treatment with antidepressant drugs on anxiety/depression parameters, and investigated whether these drugs may exert their effect on glutamate system, thereby reversing the PRS-induced impairment in glutamate release.
b) We examined the impact of sex in circadian patterns of locomotor activity.
c) We studied the impact of sex hormones on anxiety and depression in PRS male and female and their respective controls.

In Chapter 2, we wanted to move further in the characterization of PRS enhanced sensitivity to addiction by taking into account the addictive properties of chocolate as natural reward and the impact of gender and sex hormones in shaping the hedonic sensitivity to natural reward and vulnerability to drug addiction.

a) We studied the response of PRS male and female rats and the respective unstressed control animals to palatable food in CPP. We then assessed the influence of sex hormones in such response. We also evaluated the expression of key genes in the hypothalamus and monoamines steady-state levels in the NAc and prefrontal cortex.
b) We evaluated the response to cocaine in the CPP paradigm both in male and female PRS and unstressed control rats.

In Chapter 3, we addressed whether a behavioral sensitizing history of cocaine could have a beneficial impact on the anxious-/depressive-like profile and on the alterations of the glutamatergic system induced by PRS.

Overall, my thesis aims at strengthening the finding that PRS is an attractive model to study the comorbidity between anxious-/depressive-like disorders and addiction and that sex/sex hormones have a predominant role in shaping the influence of early life stress.

RESULTS

CHAPTER I: PRS AND SEX DIFFERENCES ON ANXIETY AND DEPRESSION

1- *The effects of antidepressant treatment in prenatally stressed rats support the glutamatergic hypothesis of stress-related disorders*

Data from a recent work highlight the key role of the ventral hippocampus in the pathophysiology of the anxiety-like phenotype induced by PRS (Marrocco et al., 2012). A deficit in glutamate release specifically in the ventral hippocampus (which specifically encodes memory related to emotions, Fanselow and Dong, 2010) of PRS rats lies at the core of the behavioral alterations observed in these animals, and the pharmacological enhancement of glutamate release by a mixture of drugs blocking mGlu2/3 receptors and GABAB receptors, known to negatively regulate glutamate release in the hippocampus (reviewed by Chalifoux and Carter, 2011; Nicoletti et al., 2011) was able to correct the anxiety-like behavior in PRS rats (Marrocco et al., 2012).

As aforementioned, antidepressant drugs reverse several PRS-induced alterations at the behavioral, neurochemical and neuroanatomical levels (Morley-Fletcher et al., 2011; Mairesse et al., 2013). In the present study, we wanted to assess whether the deficit in glutamate release and the glutamatergic hypofunction in PRS rats could be restored by chronic treatment with the antidepressant drugs agomelatine and fluoxetine, in relation to the anxiety- and depressive-like phenotype.

Behavioral/Cognitive

The Effects of Antidepressant Treatment in Prenatally Stressed Rats Support the Glutamatergic Hypothesis of Stress-Related Disorders

Jordan Marrocco,[1] Marie-Line Reynaert,[2,6] Eleonora Gatta,[2,6] Cecilia Gabriel,[3] Elisabeth Mocaër,[3] Silvia Di Prisco,[4] Elisa Merega,[4] Anna Pittaluga,[4] Ferdinando Nicoletti,[5,6] Stefania Maccari,[2,6] Sara Morley-Fletcher,[2,6]* and Jérôme Mairesse[2,6]*

[1]Istituto di Ricovero e Cura a Carattere Scientifico, Centro Neurolesi "Bonino-Pulejo," Messina 98124, Italy, [2]Neural Plasticity Team-Unité Mixte de Recherche 8576 Centre National de la Recherche Scientifique/Université Lille 1, Department of Structural and Functional Glycobiology, F-59655 Villeneuve d'Ascq, France, [3]Institutes de Recherches Internationales Servier, 92150 Suresnes, France, [4]Department of Pharmacy, University of Genoa, Genoa 16146, Italy, [5]Sapienza University of Rome and Istituto di Ricovero e Cura a Carattere Scientifico Neuromed, Pozzilli 86077, Italy, and [6]International Associated Laboratory-Prenatal Stress and Neurodegenerative Diseases, F-59655 Villeneuve d'Ascq, IT-00185 Rome, IT-86077 Pozzilli

Abnormalities of synaptic transmission in the hippocampus represent an integral part of the altered programming triggered by early life stress, which enhances the vulnerability to stress-related disorders in the adult life. Rats exposed to prenatal restraint stress (PRS) develop enduring biochemical and behavioral changes characteristic of an anxious/depressive-like phenotype. Most neurochemical abnormalities in PRS rats are found in the ventral hippocampus, a region that encodes memories related to stress and emotions. We have recently demonstrated a causal link between the reduction of glutamate release in the ventral hippocampus and anxiety-like behavior in PRS rats. To confer pharmacological validity to the glutamatergic hypothesis of stress-related disorders, we examined whether chronic treatment with two antidepressants with different mechanisms of action could correct the defect in glutamate release and associated behavioral abnormalities in PRS rats. Adult unstressed or PRS rats were treated daily with either agomelatine (40 mg/kg, i.p.) or fluoxetine (5 mg/kg, i.p.) for 21 d. Both treatments reversed the reduction in depolarization-evoked glutamate release and in the expression of synaptic vesicle-associated proteins in the ventral hippocampus of PRS rats. Antidepressant treatment also corrected abnormalities in anxiety-/depression-like behavior and social memory performance in PRS rats. The effect on glutamate release was strongly correlated with the improvement of anxiety-like behavior and social memory. These data offer the pharmacological demonstration that glutamatergic hypofunction in the ventral hippocampus lies at the core of the pathological phenotype caused by early life stress and represents an attractive pharmacological target for novel therapeutic strategies.

Introduction

A growing body of evidence suggests that abnormalities in hippocampal glutamatergic transmission are involved in the pathophysiology of mood and anxiety disorders (Tordera et al., 2007; Ongür et al., 2008; Garcia-Garcia et al., 2009; Chen et al., 2010; Musazzi et al., 2010, 2013; Popoli et al., 2011).

We were able to demonstrate a direct relationship between hippocampal glutamate release and anxiety in rats subjected to prenatal restraint stress (PRS; Marrocco et al., 2012). PRS rats (i.e., the offspring of mothers exposed to repeated episodes of restraint stress during the last 10 d of pregnancy) are characterized by a prolonged corticosterone response to acute stress, and by neurochemical and behavioral abnormalities that are typically linked to depression and anxiety (Dugovic et al., 1999; Darnaudéry and Maccari, 2008; Zuena et al., 2008; Morley-Fletcher et al., 2011; Mairesse et al., 2013). We found large reductions in depolarization-evoked glutamate release and in the expression of synaptic vesicle-associated proteins in the ventral hippocampus of adult PRS rats. In these rats, pharmacological enhancement of glutamate release by local injection of a cocktail of $GABA_B$ and mGlu2/3 receptor antagonists in the ventral hippocampus was able to reverse anxiety-like behavior (Marrocco et al., 2012). This was the first evidence of a hypofunction of glutamatergic neurotransmission in the ventral hippocampus in a model of depres-

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*S.M.-F. and J. Mairesse are co-last authors.

C.G. and E. Mocaër are employed by Servier. The other authors declare no competing financial interests.

Correspondence should be addressed to Prof. Stefania Maccari, LIA-Prenatal Stress and Neurodegenerative Diseases (PSND), North University of Lille, France, Neuroplasticity Team, CNRS UMR 8576/ UGSF, Structural and Functional Glycobiology Unit, Bâtiment C9, Avenue Mendeleiev, 59655 Villeneuve d'Ascq, France. E-mail: stefania.maccari@univ-lille1.fr.

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sion and anxiety, which has predictive, face, and construct validity. Glutamatergic hypofunction has also been found in postmortem brain tissue from depressed subjects (Choudary et al., 2005; Bernard et al., 2011). Of note, exposure to acute or chronic stress in adult life results instead in enhanced glutamate release in the hippocampus (Popoli et al., 2011), suggesting that the age window of exposure is critical for the effect of chronic stress on glutamatergic transmission. The evidence that the ventral portion of the hippocampus specifically encodes memories related to stress and emotions (Fanselow and Dong, 2010) strengthens the relation between our findings in PRS rats and the pathophysiology of anxiety/depressive disorders.

To support the glutamatergic hypothesis of stress-related disorders, it becomes fundamental to demonstrate that drugs that are currently used in the treatment of anxiety/depressive disorders can correct the defect in glutamate release found in the ventral hippocampus of PRS rats. To address this question, we used fluoxetine and agomelatine, two antidepressants that display different mechanisms of action. Fluoxetine is a selective serotonin reuptake inhibitor, which is marketed for the treatment of major depression, panic disorders, and obsessive-compulsive disorders (Sommi et al., 1987; Stokes and Holtz, 1997). Agomelatine is approved for the treatment of major depression and acts as a mixed MT_1/MT_2 melatonergic receptor agonist/5-HT_{2C} receptor antagonist (de Bodinat et al., 2010). Preclinical and clinical evidence demonstrate that agomelatine is also effective in the treatment of anxiety (Millan et al., 2005; Tuma et al., 2005; Loiseau et al., 2006; Papp et al., 2006; Stein et al., 2008, 2012; Baldwin and Lopes, 2009; Kasper et al., 2010; Morley-Fletcher et al., 2011; Levitan et al., 2012).

We examined the glutamatergic synapse by measuring K^+-evoked glutamate release from superfused synaptosomes and by analyzing the synaptic expression of vesicle-associated membrane protein (VAMP) as a representative protein of the SNARE complex, and of proteins regulating the trafficking of synaptic vesicles, such as synapsins, munc-18, and Rab3A (see also Marrocco et al., 2012). We report that chronic systemic treatment with either fluoxetine or agomelatine corrects the glutamatergic hypofunction in the ventral hippocampus and the associated behavioral abnormalities in adult PRS rats.

Materials and Methods

Animals

Forty nulliparous female Sprague Dawley rats (20 for control group and 20 for PRS group), weighing ~250 g, were purchased from Charles River and housed under standard conditions with a 12 h light/dark cycle. Females were individually housed overnight with a sexually experienced male rat, and vaginal smears were examined on the following morning. The day at which the smear was sperm positive was considered as embryonic day 0.

Stress protocol

Animals were subjected to PRS according to our standard protocol (Maccari et al., 1995; Morley-Fletcher et al., 2003). From day 11 of pregnancy until delivery, pregnant female rats were subjected to three stress sessions daily (45 min each), during which they were placed in transparent plastic cylinders and exposed to bright light. Only male offspring from litters containing 10–14 pups with a comparable number of males and females were used for the experiments. A maximum of one or two male pups was taken from each litter for each measure to remove any litter effects (Becker and Kowall, 1977; Chapman and Stern, 1979). All experiments followed the rules of the European Communities Council Directive 2010/63/EU of 22 September 2010. The local ethics committee approved the prenatal stress procedure.

Antidepressant treatment

Antidepressant drugs were dissolved in hydroxyethylcellulose (HEC; 1% suspension in distilled water). Rats were 3 months old at the beginning of the treatment. Control and PRS rats were treated daily over 3 weeks with intraperitoneal injections of fluoxetine (5 mg/ml/kg; Sigma), agomelatine (40 mg/2 ml/kg; Servier), or 1 ml/kg HEC alone (vehicle). The dose of agomelatine was selected on the basis of previous reports (Van Reeth et al., 1997; Papp et al., 2003; Banasr et al., 2006; Soumier et al., 2009) and on previous data obtained in the PRS rats (Morley-Fletcher et al., 2011). The dose of fluoxetine was administered according to the standard regimen described by Nibuya et al. (1996). Injections were performed 2 h before the onset of the dark phase of the 12 h light/dark cycle, based on the circadian rhythm resynchronization properties and antidepressant activity of agomelatine (Van Reeth et al., 1997; Papp et al., 2003).

All animals used for *ex vivo* measurements of neurotransmitter release and immunoblot analysis of protein expression had been tested for behavior at least 1 week earlier.

Glutamate and GABA release experiments

Purified synaptosomes isolated from the ventral hippocampus ($n = 5$ per group) were prepared essentially according to the procedures of Dunkley et al. (1986), with minor modifications. Briefly, the tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris (final concentration, 0.01 M) using a glass Teflon tissue grinder (clearance, 0.25 mm). The homogenate was centrifuged at 1000 × *g* for 5 min to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll gradient (6%, 10%, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 × *g* for 5 min. The layer between 10% and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. The synaptosomal pellet was then resuspended in physiological medium (standard medium) having the following composition (in mM): NaCl 140; KCl 3; $MgSO_4$ 1.2; $CaCl_2$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$ 5; HEPES 10; and glucose 10; pH 7.2–7.4. Synaptosomes were incubated for 15 min at 37°C in a rotary water bath and superfused at 0.5 ml/min with standard physiological solution. When studying the release of neurotransmitter evoked by high concentrations of K^+, synaptosomes were transiently (90 s) exposed, at $t = 39$ min, to 12 mM KCl (substituted for NaCl in the superfusate). Superfusion was always performed with media containing 50 μM amino-oxyacetic acid (Sigma) to inhibit GABA metabolism. Three superfusate fractions were collected according to the following scheme: two 3 min fractions (basal release), one before ($t = 36$–39 min; b1) and one after ($t = 45$–48 min; b3) a 6 min fraction ($t = 39$–45 min; evoked release, b2). Fractions collected and superfused synaptosomes were counted for endogenous amino acid content. Endogenous glutamate and GABA were measured by HPLC analysis after pre-column derivatization with *o*-phthalaldehyde and separation on a C18 reverse-phase chromatographic column (10 × 4.6 mm, 3 μm; at 30°C; Chrompack) coupled to fluorimetric detector (excitation wavelength, 350 nm; emission wavelength, 450 nm). Buffers and the gradient program were as follows: solvent A, 0.1 M sodium acetate, pH 5.8/methanol, 80:20; solvent B, 0.1 M sodium acetate, pH 5.8/methanol, 20:80; solvent C, 0.1 M sodium acetate, pH 6.0/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; isocratic flow, 2 min; 78% solvent A and 22% solvent B in 2 min; isocratic flow, 6 min; 66% solvent A and 34% solvent B in 3 min; 42% solvent A and 58% solvent B in 1 min; 100% solvent B in 1 min; isocratic flow, 2 min; 100% solvent C in 3 min; flow rate, 0.9 ml/min. Homoserine was used as the internal standard. Synaptosomal protein contents were determined according to Bradford (1976). The amount of endogenous glutamate and GABA from synaptosomes in superfusate fractions was expressed as picomoles per milligram of protein. The depolarization-induced overflow was estimated by subtracting the neurotransmitter content into the first and the third 3 min fractions collected (basal release, b1 and b3) from that in the 6 min fraction collected during and after the depolarization pulse (evoked release, b2).

Western blot analysis

Control and PRS rats ($n = 4$ per group) were killed by decapitation, and dorsal and ventral hippocampi were rapidly dissected and immediately

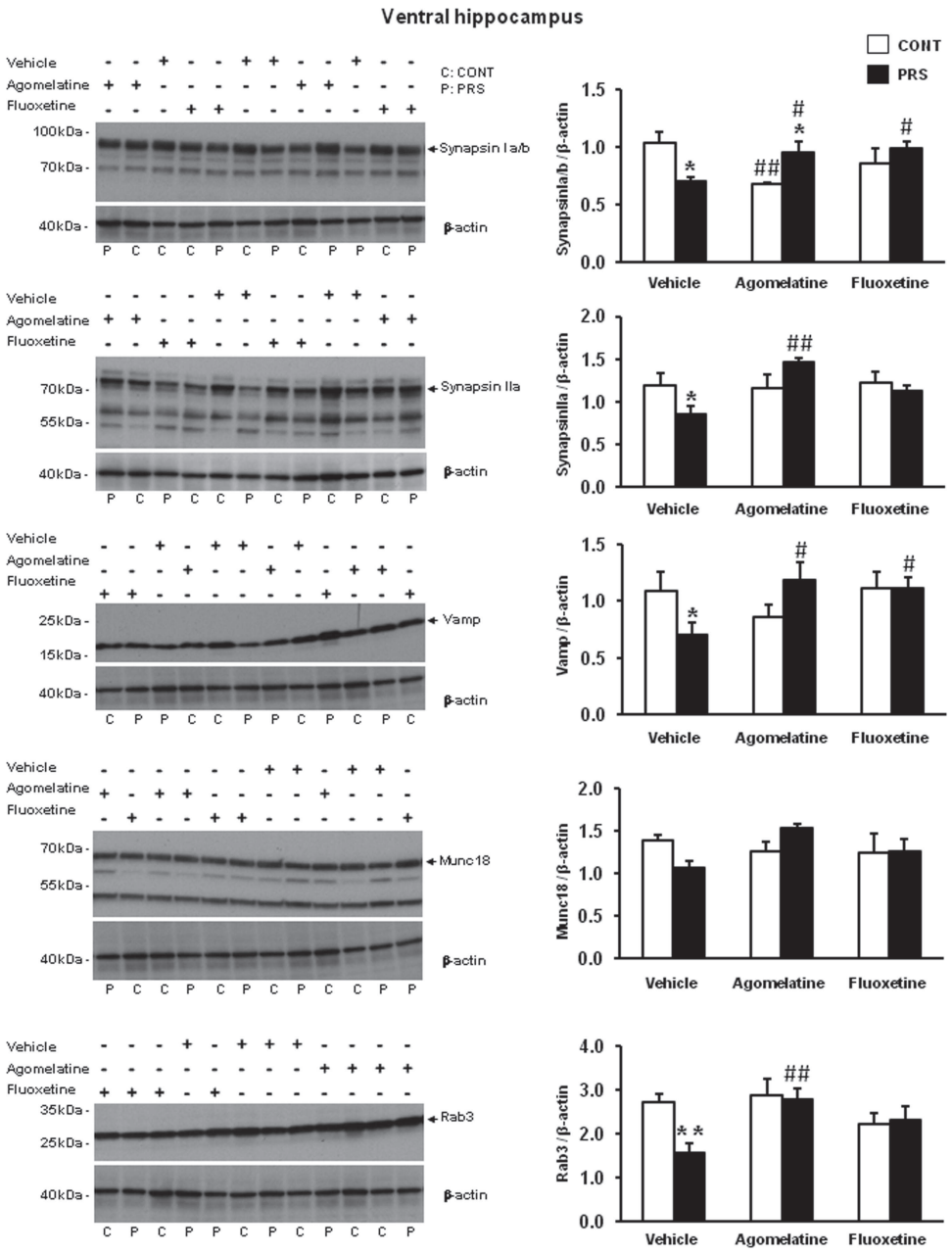

Figure 1. Chronic treatment with agomelatine or fluoxetine corrects abnormalities in the expression of synaptic vesicle-associated proteins in the ventral hippocampus of PRS rats. Control unstressed rats (CONT) and PRS rats were treated intraperitoneally with vehicle, agomelatine (40 mg/kg), or fluoxetine (5 mg/kg) for 21 d. Representative continuous, uncropped images of 12 sample immunoblots (2 samples per group per treatment) are shown on the left side (C, control unstressed rats; P, PRS rats). Values are given as the mean ± SEM ($n = 4$ rats per group). *$p < 0.05$ or **$p < 0.01$ vs the respective CONT rats; #$p < 0.05$ or ##$p < 0.01$ vs vehicle-treated CONT or PRS rats.

stored at −80°C. Immunoblotting analysis was performed on the synaptosomes isolated from the ventral hippocampus. To isolate synaptosomes, tissue was manually homogenized with a potter in 10 volumes of HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES pH 7.4). All procedures were performed at 4°C. Homogenates were centrifuged at 1000 × *g* for 10 min, and the resulting supernatants were centrifuged at 10,000 × *g* for 15 min. The pellet obtained from the second centrifugation was resuspended in 10 volumes of HEPES-buffered sucrose and then spun again at 10,000 × *g* for 15 min. This pellet contained the crude synaptosomal fraction. To validate the purity of this synaptosomal fraction, we used anti-histone H3, anti-β-tubulin, and anti-synapsin Ia/b in immunoblot analysis. BCA assay was used to determine protein concentration. Synaptosome lysates were resuspended in Laemmli reducing buffer, and 20 μg of each sample was first separated by electrophoresis on Criterion TGX 4–15% precast SDS-PAGE gels (26 wells; Bio-Rad) and later transferred to nitrocellulose membranes (Bio-Rad). Transfer was performed at 4°C in a buffer containing 35 mM Tris, 192 mM glycine, and 20% methanol.

We used the following primary antibodies: rabbit polyclonal anti-synapsin Ia/b (1:4000; catalog #sc-20780), rabbit polyclonal anti-synapsin IIa (1:4000; catalog #sc-25538) and rabbit polyclonal anti-VAMP (1:2000; synaptobrevin; catalog #sc-13992), all purchased from Santa Cruz Biotechnology; mouse monoclonal anti-rab3a (1:2000; catalog #107111) and mouse monoclonal anti-Munc-18 (1:2000; catalog #116011), which were purchased from Synaptic Systems. All primary antibodies were incubated overnight at 4°C. HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (purchased from GE Healthcare) were used at a dilution of 1:10000 and were incubated for 1 h at room temperature. Densitometric analysis was performed with Quantity One software (Bio-Rad) associated with a GS-800 scanner. The ratio of indi-

vidual proteins to β-actin was then determined, and these values were compared for statistical significance.

Behavioral analysis

Assessment of social memory

At day 15 of antidepressant treatment, the juvenile recognition abilities of the rats ($n = 9$ per group) were assessed using the procedure described by Dantzer et al. (1987) and adapted as follows. During each of the three sessions (three sessions per day), a given juvenile rat (handled with rubber gloves) was introduced into the home cage of the adult rat for 5 min in a normally illuminated, quiet room during the light phase of the cycle (i.e., between 3:30 and 6:30 P.M.). Then, the juvenile was removed, kept individually in a cage (39 × 24 × 16 cm) with fresh bedding and food and water available *ad libitum* for a defined interexposure interval of 30 or 120 min; it was then presented again to the adult for a 5 min period. Sessions were video recorded, and the times spent in sniffing (the tested animal sniffs the challenger's fur), grooming (licking behavior of the tested animal toward the challenger), ano-genital interaction (the tested animal sniffs challenger's ano-genital zone), and play (the tested animal is in rearing position and interacts with anterior paws with the challenger) were measured by a trained observer (Observer 20, Noldus).

Assessment of anxiety-like behavior

Anxiety-like behavior was assessed on day 16 of chronic antidepressant treatment in the light and dark test as previously described (Marrocco et al., 2012). The light and dark box setup consisted of the following two compartments: one light compartment (45 × 32 × 32 cm; 50 lux; light box) and one dark compartment (30 × 32 × 32 cm; 5 lux). The compartments were connected via a small opening (10 × 15 cm) enabling transition between the two boxes. Rats ($n = 9$ per group) were placed in the light compartment, and the time spent in each compartment and the latency to the first entry into the light compartment during the 5 min test were assessed on-line via a video camera located above the box. Behavior was automatically analyzed using video-tracking software (View Point).

Assessment of depressive-like behavior

Forced swim test. At day 18 of antidepressant treatment, rats ($n = 9$ per group) were subjected to an adapted version of the forced swim test (Porsolt et al., 1978) in a cylindrical container (height, 59 cm; diameter, 25 cm) filled with water at 25°C up to a level of 36 cm. The test was performed between 12:00 and 5:00 P.M. Twenty-four hours after a 15 min session (pretest, on day 15), control and PRS rats were tested (day 16) during a 5 min session, during which immobility latency and duration, climbing, and swimming were automatically analyzed using video-tracking software (View Point).

Splash test. At day 19 of treatment, a separate set of animals ($n = 6–8$ per group) underwent an adapted version of the splash test (Santarelli et al., 2003; Yalcin et al., 2005; Surget et al., 2008). Briefly, the test consisted of spraying a 10% sucrose solution on the rat in a familiar cage. The sucrose solution dirtied the coat and induced a grooming behavior. After applying sucrose solution, the time spent grooming was recorded for 5 min as an index of self-care and motivational behavior. Previous works in mice have shown that in the splash test, chronic stress decreases grooming behavior, a form of motivational behavior considered to parallel apathetic behavior as a symptom in depression (Isingrini et al., 2010). Moreover, stress-induced grooming perturbation is associated with reduced hedonic reactivity in the sucrose preference test and increased immobility in the forced swim test (Pothion et al., 2004; Isingrini et al., 2010).

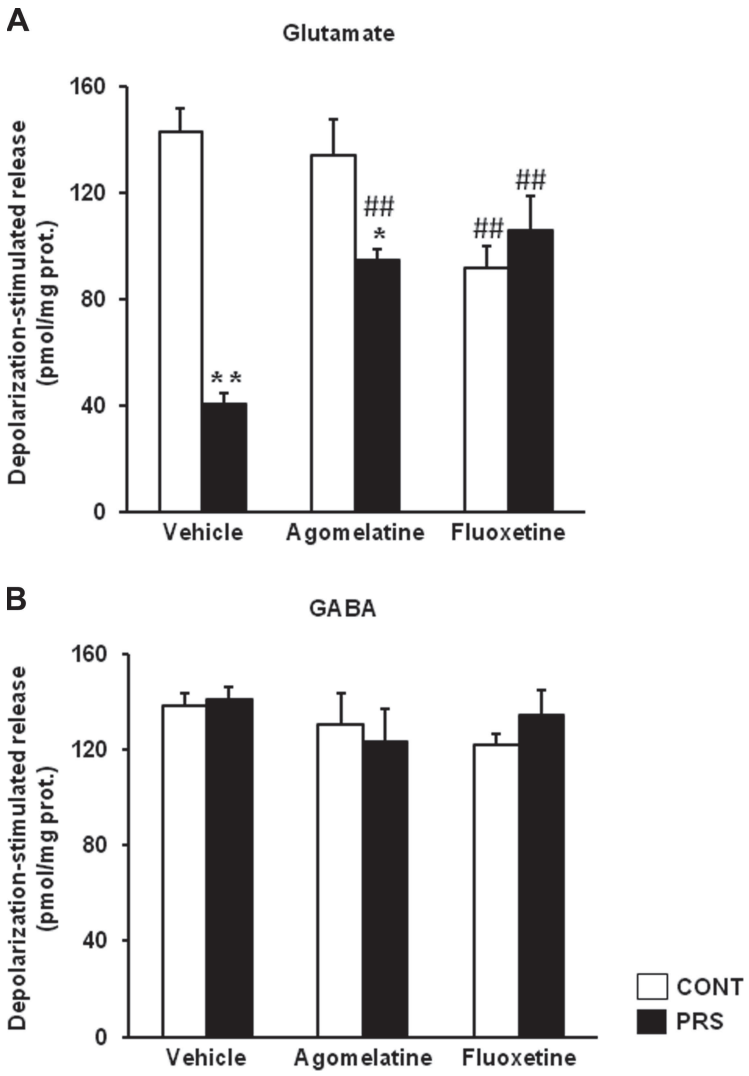

Figure 2. Chronic treatment with agomelatine or fluoxetine largely restores glutamate release in synaptosomes prepared from the ventral hippocampus of PRS rats. ***A***, ***B***, Depolarization-evoked glutamate (***A***) or GABA (***B***) release was assessed in superfused synaptosomes prepared from control unstressed rats (CONT) and PRS rats treated with vehicle, agomelatine (40 mg/kg), or fluoxetine (5 mg/kg) for 21 d. Values are given as the mean ± SEM ($n = 5$ rats per group). *$p < 0.05$ or **$p < 0.01$ vs the respective CONT rats; ##$p < 0.01$ vs vehicle-treated CONT or PRS rats.

Statistical analysis

Data were analyzed by two-way ANOVA (group by treatment) with the exception of data on social memory, which were analyzed by three-way ANOVA for repeated measures (group × treatment × interval). The Fisher's least significant difference *post hoc* test was used to isolate the differences. Correlations were analyzed using the Pearson's correlation analysis. A p value ≤0.05 was considered to be statistically significant.

Results

Chronic treatment with antidepressants reverses the changes in synaptic vesicle proteins and the ensuing defect in glutamate release in the ventral hippocampus of PRS rats

We measured the levels of synaptic vesicle proteins in purified synaptosomal membranes prepared from the ventral hippocam-

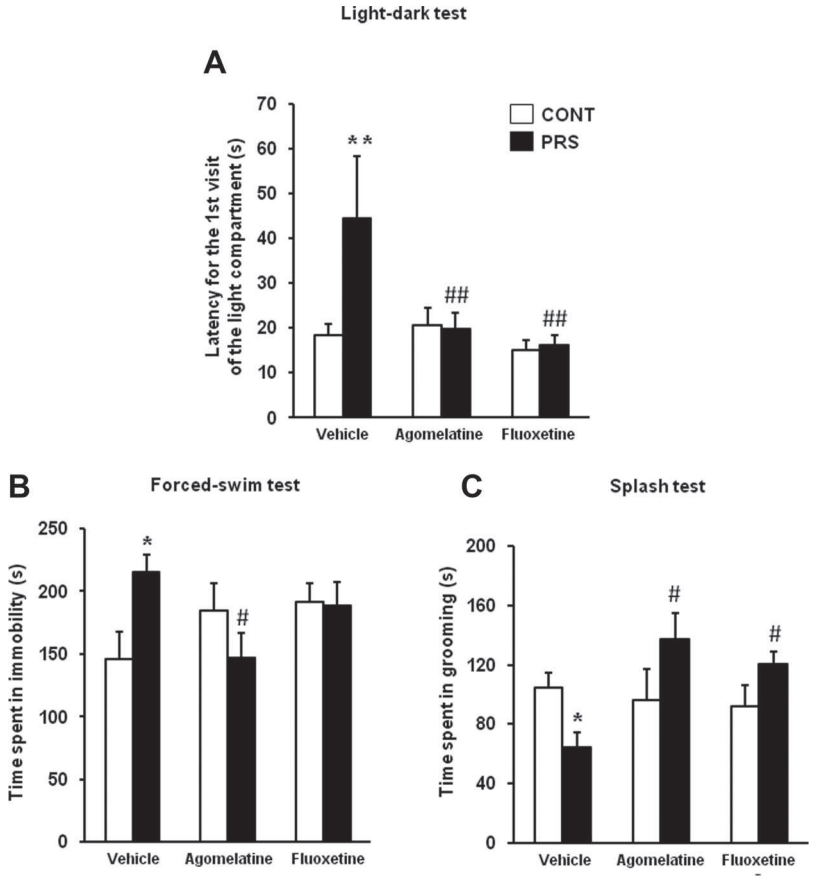

Figure 3. Chronic treatment with agomelatine or fluoxetine corrects anxiety- and depression-like behaviors in PRS rats. ***A***, ***B***, The same groups of rats used for the assessment of glutamate release were examined in the light-dark box (***A***), and in the forced swim test (***B***), as indicated in the Methods session. Different groups of rats were tested in the splash test (***C***). Control unstressed rats (CONT) and PRS rats were treated intraperitoneally with vehicle, agomelatine (40 mg/kg), or fluoxetine (5 mg/kg) for 21 d. Values are given as the mean ± SEM ($n = 9$ rats per group). *$p < 0.05$ or **$p < 0.01$ vs the respective CONT rats; #$p < 0.05$ or ##$p < 0.01$ vs vehicle-treated CONT or PRS rats.

pus of control and PRS rats. PRS rats treated with vehicle for 21 d showed significant reductions in the levels of synapsin Ia/b (group × treatment, $F_{(2,18)} = 6.87$, $p < 0.01$), synapsin IIa (group × treatment, $F_{(2,18)} = 4.25$, $p < 0.05$), VAMP (synaptobrevin; group × treatment, $F_{(2,18)} = 3.55$, $p = 0.05$), and Rab3A (group × treatment, $F_{(2,18)} = 3.54$, $p = 0.05$), and a trend to a reduction in munc18 compared with control unstressed rats treated with vehicle (Fig. 1; see also Marrocco et al., 2012). Chronic treatment with either agomelatine (40 mg/kg/d, i.p.) or fluoxetine (5 mg/kg/d, i.p.) for 21 d normalized the levels of synaptic vesicle-associated proteins in PRS rats. After agomelatine treatment, levels of synapsin Ia/b were higher in PRS than in control unstressed rats, but this was due to the lowering effect of agomelatine on synapsin Ia/b in control rats. No main changes due to PRS or antidepressant treatment were observed in synaptic proteins in the dorsal hippocampus (data not shown). Glutamate and GABA release was measured in synaptosomes using a superfusion method that allows a clean estimation of Ca^{2+}-dependent glutamate exocytosis without the components mediated by the endogenous activation of either presynaptic autoreceptors/heteroreceptors or membrane transporters (Raiteri et al., 1974; Raiteri and Raiteri, 2000; Bonanno et al., 2005). Synaptosomes prepared from the ventral hippocampus of unstressed control or PRS rats treated with vehicle, agomelatine, or fluoxetine were challenged with depolarizing concentrations of K^+, and the superfusate was used for measurements of endogenous glutamate and GABA release. Basal glutamate release did not change as a function of groups (data are expressed as pmoles/milligram protein in the first and the third 3 min fractions; control rats treated with vehicle: 156.12 ± 13.66; PRS rats treated with vehicle: 137.82 ± 17.64) or treatments (control rats treated with agomelatine: 147.54 ± 17.5; control rats treated with fluoxetine: 172.14 ± 22.31; PRS rats treated with agomelatine: 134 ± 21.21; PRS rats treated with fluoxetine: 154 ± 12.37). In contrast, depolarization-evoked glutamate release (i.e., depolarization-induced overflow) was substantially reduced in hippocampal synaptosomes of PRS rats treated with vehicle, compared with the respective control group (ANOVA group × treatment, $F_{(2,24)} = 18.67$, $p < 0.01$; Fig. 2*A*). This reduction was corrected in PRS rats treated with agomelatine and fluoxetine ($p < 0.01$). No difference in depolarization-evoked glutamate release was seen between PRS and control rats treated with fluoxetine. However, this datum is biased by the reduction of glutamate release found in control rats treated with fluoxetine ($p < 0.01$). Depolarization-evoked glutamate release was significantly higher in PRS rats treated with agomelatine or fluoxetine, compared with PRS rats treated with vehicle ($p < 0.05$). No changes in basal and depolarization-evoked GABA release were observed in control and PRS rats treated with vehicle or antidepressants (Fig. 2*B*).

Thus, chronic treatment with antidepressants normalizes both vesicle-associated proteins and depolarization-evoked glutamate release in the ventral hippocampus of PRS rats, with no or slight effect on control unstressed rats.

Behavioral effects of agomelatine or fluoxetine treatments

We used the light-dark test and the forced swim test for the assessment of anxiety-like behavior and depressive-like behavior in PRS rats (Morley-Fletcher et al., 2011; Marrocco et al., 2012). We also used the splash test for the assessment of self-care and hedonic behavior (Surget et al., 2008). In addition, we examined social recognition toward a juvenile rat as a test for social memory (Dantzer et al., 1987). The same groups of animals used for measurements of glutamate release underwent a social memory test at day 15, anxiety-like behavior test at day 16, and forced swim test at day 18 of drug treatment. A separate group of rats was used for the splash test at day 19 of treatment.

PRS rats treated with vehicle displayed an increased latency to enter the light compartment of the light-dark box, as expected. Both agomelatine and fluoxetine abolished differences in anxiety-like behavior between control and PRS rats (ANOVA, group × treatment, $F_{(2,48)} = 4.95$, $p < 0.05$; Fig. 3*A*). The action of agomelatine and fluoxetine diverged in the two tests used for the assessment of depressive-like behavior. In the forced swim test, agomelatine, but not fluoxetine, reduced the increased immobility time in PRS rats (ANOVA, group × treatment, $F_{(2,48)} =$

4.24, $p < 0.05$; Fig. 3*B*). PRS rats showed reduced grooming behavior in the splash test, which reflects an impaired motivation, and treatment with both agomelatine and fluoxetine reversed this type of depressive-like behavior (ANOVA, group × treatment, $F_{(2,37)} = 4.49$, $p < 0.05$; Fig. 3*C*). Finally, we assessed cognitive social performance by examining the ability to recognize a juvenile challenger through three consecutive 5 min exposures. In unstressed control rats, sniffing behavior was reduced from the first to the second and third exposure to the intruder. Sniffing behavior was reduced to a lesser extent in PRS rats, and, again, this behavioral abnormality was corrected by treatments with fluoxetine or agomelatine (ANOVA, group × treatment × interval exposure, $F_{(4,96)} = 2.51$, $p < 0.05$; Fig. 4*A*,*B*).

Thus, the effect of antidepressants on anxiety- and depression-like behaviors was "disease dependent," being selectively observed in PRS rats.

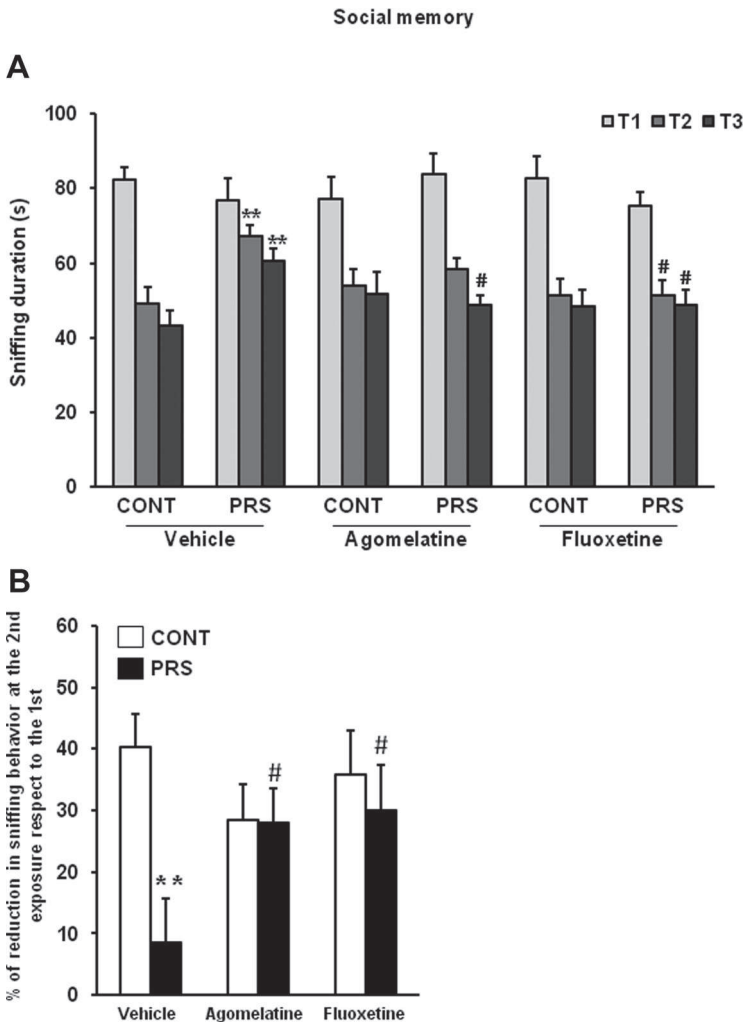

Figure 4. Chronic treatment with agomelatine or fluoxetine improves social memory in PRS rats. Control unstressed rats (CONT) and PRS rats were treated intraperitoneally with vehicle, agomelatine (40 mg/kg), or fluoxetine (5 mg/kg) for 21 d. Data on sniffing behavior at the first (T1), second (T2), and third (T3) exposure to the juvenile challenger are shown in ***A***. The percentage of reduction at the second exposure respect to the first is shown in ***B***. Values are means ± SEM ($n = 9$ rats per group). $^{**}p < 0.01$ vs the respective CONT rats; $\#p < 0.05$ vs vehicle-treated CONT or PRS rats.

The anxiolytic action of agomelatine and fluoxetine is correlated to normalization of glutamate release

We have recently reported that depolarization-evoked glutamate release in the ventral hippocampus is negatively correlated with anxiety-like behavior of PRS rats (Marrocco et al., 2012). All control and PRS rats used for measurements of glutamate release in synaptosomes ($n = 5$ rats per group) had been previously tested for anxiety-like behavior in the light-dark box, and depressive-like behavior in the forced swim test and the social memory test (see above). We examined, for each animal in each group, the correlation between depolarization-evoked glutamate release in the ventral hippocampus and (1) the latency to enter the light box; (2) the immobility time in the forced swim test; and (3) the reduction in sniffing behavior at the second exposure to the juvenile challenger. In addition, we examined whether treatment with fluoxetine or agomelatine could affect these correlations. We found that depolarization-evoked glutamate release in the ventral hippocampus was negatively correlated with anxiety-like behavior, and the correlation was maintained when the analysis included rats treated with agomelatine and fluoxetine. In contrast, glutamate release showed a positive correlation with social memory performance only when rats treated with vehicle and agomelatine were included in the analysis. Depressive-like behavior in the forced swim test showed no apparent correlation with glutamate release in the ventral hippocampus. Finally, there was no correlation among the three different behaviors with the exception of a negative correlation between social memory performance and depressive-like behavior restricted to vehicle- and agomelatine-treated rats (Fig. 5; Table 1).

Discussion

We have shown that chronic treatment with two antidepressants endowed with different mechanisms of action (i.e., fluoxetine and agomelatine) reversed the reduction in depolarization-evoked glutamate release in the ventral hippocampus and corrected a range of pathological behaviors in PRS rats. These included anxiety-like behavior in the light-dark box; increased immobility time in the forced swim test; reduced grooming behavior in the splash test, reflecting low self-care; and reduced social memory performance toward a juvenile challenger. The effects of fluoxetine and agomelatine were similar, but not identical. In general, agomelatine showed a more complete profile than fluoxetine in correcting the neurochemical and behavioral abnormalities of PRS rats, with poor or no effects in unstressed control rats. In contrast, fluoxetine treatment abolished most of the differences between unstressed and PRS rats, but also caused a small but significant reduction of glutamate release in the ventral hippocampus of unstressed rats. Thus, at least in our model, agomelatine behaves as a "disease-dependent" drug, being selective for the pathological state (see also Morley-Fletcher et al., 2011). The lack of agomelatine effect in our control rats is in disagreement with recent findings showing that chronic agomelatine treatment reduces depolarization-evoked glutamate release in hippocampal synaptosomes of unstressed rats (Milanese

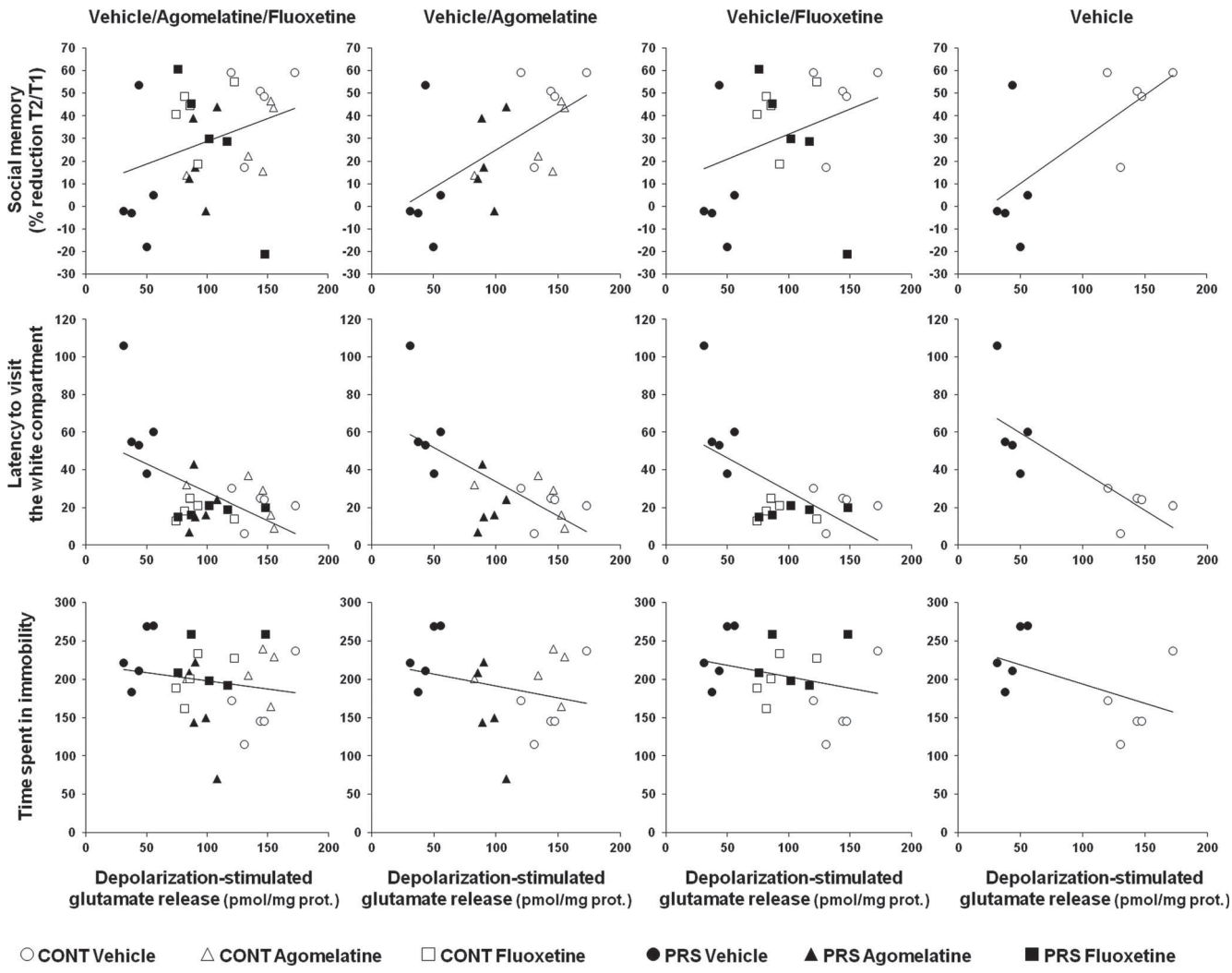

Figure 5. Correlation analysis of depolarization evoked glutamate release in the ventral hippocampus toward anxiety-like behavior in the light-dark box, depression-like behavior in the forced swim test, and social memory performance. Pearson's correlation coefficient (*r*) values and related *p* values are reported in Table 1 ($n = 5$ rats per group). CONT, Control unstressed rats.

Table 1. Statistical analysis of correlation data among depolarization-evoked glutamate release in the ventral hippocampus, anxiety-like behavior in the light-dark box, depression-like behavior in the forced-swim test, and social memory performance

	Veh/Ago/Flx		Veh/Ago		Veh/Flx		Veh	
Interactions	*r* value	*p* value	*r* value	*p* value	*r* value	*p* value	*r* value	*p* value
Glutamate release × social memory	0.32	0.08	0.62*	0.004	0.34	0.14	0.70*	0.024
Glutamate release × anxiety-like behavior	−0.49*	0.005	−0.58*	0.007	−0.54*	0.013	−0.78*	0.007
Glutamate release × depressive-like behavior	−0.17	0.36	−0.26	0.26	−0.28	0.22	−0.51	0.13
Anxiety-like behavior × social memory	−0.30	0.11	−0.29	0.21	−0.37	0.10	−0.47	0.16
Anxiety-like behavior × depressive-like behavior	0.15	0.42	0.25	0.27	0.22	0.36	0.50	0.14
Social memory × depressive-like behavior	−0.31	0.08	−0.47*	0.036	−0.37	0.10	−0.40	0.24

Data represent Pearson's correlation coefficient (*r*) and related *p* values. The level of significance was set at $p < 0.05$ (*). Veh, Vehicle; Ago, agomelatine; Flx, fluoxetine.

et al., 2013). The following factors might contribute to explaining these contrasting findings: (1) the different breeding of the animals (reared from birth in the animal facility in our study); (2) the execution of multiple behavioral tasks before the assessment of glutamate release in our study; (3) use of ventral versus total hippocampus in the two studies; and (4) the different concentrations of K^+ ions used to stimulate glutamate release (12 vs 15 mM), resulting in different extents of depolarization-evoked release in the two studies.

So far, the pharmacological validity of the glutamatergic hypothesis of anxious/depressive disorders was mainly supported by the antidepressant activity of ketamine, which behaves as a slow NMDA receptor channel blocker (for review, see Maeng and Zarate, 2007). To date, the effects of classic antidepressants on glutamate release have been investigated either under basal conditions or in response to acute or chronic stress that induces a hyperfunction of glutamatergic neurotransmission in adult "normal" rats (Bonanno et al., 2005; Musazzi et al., 2010; Tardito et al., 2010; Reagan et al., 2012; Milanese et al., 2013). Adult animals exposed to acute or chronic stress represent a model of reactive depression or post-traumatic stress disorder. Here we were able to demonstrate an action of antidepressants on glutamatergic

transmission in PRS rats that recapitulates the hallmark features of endogenous depression and anxiety, and are characterized by a reduction in glutamate release in the ventral hippocampus (see also Marrocco et al., 2012). Antidepressant treatment in PRS rats enhanced glutamate release without changing GABA release. This action might correct the imbalance between excitatory and inhibitory neurotransmission in the ventral hippocampus, thereby restoring cognitive functions related to stress and emotions in PRS rats (Bannerman et al., 2004; Engin and Treit, 2007; Fanselow and Dong, 2010). The evidence that chronic antidepressant treatment normalizes either the increase or the decrease in glutamate release (in normal rats exposed to stress and PRS rats, respectively) suggests that the action of antidepressants critically involves glutamatergic transmission in the hippocampus.

The precise mechanism by which antidepressants modulate the function of glutamatergic synapses in the hippocampus remains unknown. The primary mechanisms of action of fluoxetine and agomelatine may converge into a common intracellular pathway leading to a functional remodeling of glutamatergic terminals. An attractive hypothesis is that, regardless of their primary mechanisms of action, antidepressants epigenetically regulate the expression of synaptic vesicle-associated proteins at glutamatergic terminals, thereby correcting the abnormalities of glutamate released caused by different forms of stress. Epigenetic mechanisms are now considered as potential targets for antidepressant medication (Nasca et al., 2013; Vialou et al., 2013), and the possibility that these mechanisms involve synaptic vesicle-associated proteins warrants in-depth investigation. It will also be interesting to examine whether agomelatine and fluoxetine act directly on glutamatergic neurons in the ventral hippocampus or modulate glutamatergic transmission transynaptically by acting primarily on other stations of the neural circuitry underlying stress and emotion.

The use of the same animals from each group for behavioral studies and *ex vivo* measurements of neurotransmitter release enabled a correlation analysis between glutamate release in the ventral hippocampus and anxiety-like behavior, depressive-like behavior in the forced swim test, and social memory performance. Interestingly, the extent of glutamate release was inversely related to anxiety, and showed a positive correlation with social memory performance when rats treated with agomelatine were included in the analysis. This particular profile of correlation was expected because (1) agomelatine was highly effective in improving both anxiety-like behavior and social memory in PRS rats, and (2) reduction of anxiety has a favorable impact on social memory (Landgraf et al., 1995) by influencing the balance between reserve and explorative curiosity and improving cognitive flexibility (Blazevic et al., 2012). It is reasonable to conclude that agomelatine decreases anxiety-like behavior in PRS rats by correcting the defect of glutamate release in the ventral hippocampus, and that the improvement in social memory is a direct consequence of the anxiolytic effect. We were surprised to find no correlation between glutamate release in the ventral hippocampus and depression-like behavior in the forced swim test, as well as between anxiety- and depression-like behaviors. We suggest that anxiety and depression are two unrelated psychopathological outcomes of the neuroadaptive programming triggered by early life stress, of which only anxiety might be directly linked to a presynaptic impairment of glutamate release in the ventral hippocampus.

In conclusion, our data provide the first pharmacological validation for the "glutamatergic hypothesis" of stress-related disorders (Holden, 2003; Hashimoto, 2009; Popoli et al., 2011; McCarthy et al., 2012) by demonstrating the action of novel and classic antidepressants in the PRS model, which replicates developmental factors involved in the etiology of anxious/depressive disorders (for review, see Krishnan and Nestler, 2010), and also has predictive and face validity as an experimental animal model of anxiety and depression in humans (Darnaudéry and Maccari, 2008). This lays the groundwork for the study of glutamate release in the ventral hippocampus in other experimental models, and in humans with anxiety and depression. We suggest that glutamatergic transmission in the ventral hippocampus, a key brain region involved in the maladaptive programming caused by early life stress, represents an attractive pharmacological target for the development of novel therapeutic strategies.

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2- *Altered circadian patterns in the prenatally restraint stress rat model of depression*

PRS adult rats present persistent changes in sleep architecture that parallel those found in depressed patients (Dugovic et al., 1999). It has been shown that PRS rats exhibited alterations in the sleep architecture: reduced duration of slow wave sleep, increased duration of rapid eye movement (REM) sleep, increased number of REM sleep events and an increase in motor activity before the beginning of the dark phase of the light/dark cycle. A chronic treatment with the ATD drug agomelatine, which was able to correct the alterations in the anxious-/depressive-like profile of PRS rats *via* a restoration of abnormalities in glutamatergic transmission, as described in the previous section, also corrected PRS-induced abnormalities in sleep architecture and restore circadian rhythms of motor activity in the PRS preclinical model of depression (Mairesse et al., 2013).

Gender is a key unmodifiable risk factor in depression and both epidemiological and clinical studies consistently find that women are much more likely than men to be diagnosed with depression, with doubled prevalence rates in comparison to men (Leach et al., 2008). We were able to demonstrate in two distinct studies that PRS males and females displayed similar depressive-like profile in the forced swim test (Morley-Fletcher et al., 2011; Van Waes et al., 2006), while a sex dimorphism was found for anxiety (Zuena et al., 2008).

Emerging evidence in both humans and rodents suggests that disturbances of the circadian system may contribute to individual differences in emotionality, and emergence of depression and anxiety or lability, impulsivity, and aggression (Germain and Kupfer, 2008; McClung, 2007). For example, alterations in the expression of Clock, one of the core circadian genes, potentiates reward drive, novelty-seeking, and impulsivity, disrupts sleep patterns, and reduces depression- and anxiety-like behaviors in mice (Le-Niculescu et al., 2008; Roybal et al., 2007), suggesting an important link between the circadian system and different aspects of emotionality. Likewise, human post-mortem studies have documented gene expression alterations within brain regions that regulate the LHPA axis, including increased corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA in the paraventricular nucleus of the hypothalamus (PVN) (Bao et al., 2008), and altered ratios of glucocorticoid (GR) and mineralocorticoid (MR) receptor mRNAs in the hippocampus of depressed patients (Lopez et al., 1998).

Here, we have studied the interplay between prenatal stress and sex in determining circadian patterns of locomotor activity, in an attempt to better characterize sex differences in depressive-like behavior inside a same study. We also investigated the circadian expression of CRH in the PRS model, in relation to the circadian pattern of locomotor activity.

Manuscript in revision

Altered circadian locomotor activity in male and female rats in the prenatal restraint stress model of depression

G. Van Camp[1], M-L. Reynaert[1], E. Gatta[1], C. Laloux[2], A. Tramutola[3], C. Cinque[2], P. Navarra[3], S. Morley-Fletcher[1], F. Nicoletti[2,4], S. Maccari[1*] and J. Mairesse[1]

1. Neuroplasticity Team, UMR 8576 CNRS, North Univ. of Lille – Lille 1, France
2. Dept. of Human Physiol. & Pharmacol., Sapienza Univ. of Rome, Italy
3. Instituto di Farmacologia, Univ. Catt. Sacro Cuore, Rome, Italy
4. IRCCS, NEUROMED, Pozzilli, Italy

***Corresponding author:**

Pr. Stefania Maccari

Neuroplasticity Team, UMR 8576 CNRS "UGSF",

North University of Lille – Lille 1, 59655, Villeneuve d'Ascq, France.

Tel number: +33 621654358; Fax number: +33 320436555

E-mail address: stefania.maccari@univ-lille1.fr

Abstract

Prenatal restraint stress (PRS) in rats is a well-documented model of early stress known to induce depression-like behavior. Interestingly, most the enduring changes induced by PRS are sex-dependent, with a depression-like phenotype being present in both males and females, and an anxiety-like phenotype predominating in males. PRS causes reductions in hippocampal neurogenesis, CREB phosphorylation and expression of mGlu5 metabotropic glutamate receptors only in male rats. In contrast, a prolonged response of the hypothalamus-pituitary-adrenal axis to stress and a reduced expression of mGlu2/3 receptors in the hippocampus are seen in both male and female PRS rats. Whether changes in circadian patterns caused by PRS are also sex-dependent is unknown. Here, we examined the relationships between PRS, gender and the circadian system by monitoring the running wheel behavior in male and female adult PRS rats, first under a regular light-dark (LD) cycle, and then after an abrupt 6h advance shift in the LD cycle. Furthermore, to investigate whether hypothalamic modifications are associated with these circadian behavioral activities, we also measured the hypothalamic CRH content in males and females at 0800h and at 2000h. The pattern of locomotor activity in PRS rats was erratic and more fragmented, particularly in female PRS rats. PRS increased and decreased total locomotor activity in males and females, respectively, and induced a significant phase advance in the rhythm of circadian activity only in males. In addition, PRS increased the time required to resynchronize the activity rhythm after an abrupt phase advance of the LD cycle to a larger extent in females than in males. At the beginning of the light phase, PRS induced a strong increase of hypothalamic CRH levels in males but not in females. At the beginning of the dark phase, PRS increased hypothalamic CRH levels in males but reduced CRH levels in females. These observations highlight the importance of the circadian system in the gender-specific outcome of PRS.

Keywords

Prenatal restraint stress; Circadian rhythm; Locomotor activity; Jet lag; CRH; Gender

Introduction

Circadian rhythms regulate various physiological events and play a major physiological role to insure optimal functioning of the organism and its adaptation to predict environmental changes (Gerstner and Yin, 2010). In particular, abnormalities in sleep and circadian rhythms are commonly observed in patients with psychiatric and neurodegenerative disorders (Wulff et al., 2010). Interestingly, mood, alertness and cognitive performance in healthy individuals have all been shown to vary with the time of the day. Mood is generally low in the morning, high in the evening, and declines with extended wakefulness. This led to the proposal that mood and cognition, like sleep and wakefulness, are partly regulated by circadian and homeostatic processes and that mood instability arises from an abnormal phase relationship between circadian and homeostatic processes (Wirz-Justice and Van den Hoofdakker, 1999; Bunney and Potkin, 2008). Despite the recognition of an association between sleep/circadian rhythm disruption and mental health, mechanistic links remain poorly understood.

Prenatal restraint stress (PRS) in rats is a well-documented animal model of depression and anxiety (Morley-Fletcher et al., 2004a,b; Maccari and Morley-Fletcher, 2007; Darnaudéry and Maccari, 2008). Interestingly, most of the outcomes of PRS appear to be gender-dependent. Early studies showed that PRS altered sex hormones only in males (Ward and Weisz, 1984; Ward, 1972). Immune stress during late pregnancy reduced anxiety-like behavior in female, but not male, rats (Paris et al., 2011). Similar findings are reported by Zuena et al. (2008) and by Brunton et al. (2011) using the model of PRS or a model of social stress during late gestation, respectively. PRS causes reductions in hippocampal neurogenesis, CREB phosphorylation and mGlu5 receptor expression only in male rats (Zuena et al., 2008). There are exceptions, however. For example, a prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress (Maccari et al., 1995) and a reduced expression of mGlu2/3 receptors in the hippocampus were seen in both male and female PRS rats, although a greater response to stress was present in females (Zuena et al., 2008).

Whether alterations in circadian patterns caused by early life stress are also sex-dependent is unknown. The PRS model is particularly appropriate to examine this question. PRS is known to increase REM sleep in male rats (Mairesse et al., 2013), and a positive correlation has been found between corticosterone response to stress and REM sleep in PRS rats (Dugovic et al., 1999). Both male and female PRS rats show an increased corticosterone secretion at the end of the light period although only female PRS rats showed an increase in total daily

corticosterone secretion (Koehl et al., 1999). Here, we monitored the running wheel behavior in male and female adult PRS rats, first under a regular light-dark (LD) cycle, and then after an abrupt 6h advance shift in the LD cycle (a chronobiological stress). We extended the analysis to hypothalamic levels of corticotrophin releasing hormone (CRH), which is involved in mechanisms regulating arousal, anxiety, and responses to stress (Steiger, 2002; Lightman, 2008). Recently, Zohar and Weinstock (2011) have found that prenatal stress differentially modulates CRH expression in male and female rats. However, CRH has never been studied in relation to circadian pattern of activity in PRS rats.

Material and methods

Animals

Sprague-Dawley nulliparous female rats weighing approximately 250g were purchased from a commercial breeder (Harlan, Italia). Animals were kept at constant temperature (22±2°C), with a regular 12h light/dark (LD) cycle (lights on at 0800h). Water and 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 placed with a sexually experienced male for a night (the following day being designated as day 0 of gestation), after which they were housed individually in Plexiglas cages (30x20x15 cm). Pregnant females were then randomly assigned to prenatal restraint stressed (PRS) or control (C) groups. (n=12 in each group).

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 subjected daily to three stress sessions starting at 0900h, 1200h and 1700h, during which they were placed in plastic transparent cylinders (diam.=7.0 cm; length=19.0 cm) and exposed to bright light for 45 min. Control pregnant females were left undisturbed in their home cages. Male and female 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. A maximum of one or two male pups were taken from each litter for each measure to remove any litter effects (Becker and Kowall, 1977; Chapman and Stern, 1979). After weaning, male and female rats

from each experimental group (Control *vs* PRS) were housed in groups of three and maintained under the same environmental conditions until experiments started (at 4 months of age). All experiments followed the rules of the European Communities Council Directive 86/609/EEC.

Running wheel activity

All rats were 4 months old at the start time of experiments. Rats were housed in light tight chambers equipped with continuously operating ventilating fans and placed in individual cages equipped 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 LD cycle (light intensity was set at 30–40 lx at cage floor level). During the course of the experiments, food and water were provided *ad libitum*, room temperature (22 °C) and humidity (60 %) were kept constant. A first group of rats (n = 10 for each group) was used to analyze the rhythm of circadian activity under a regular 12/12 LD cycle.

After 10–15 days of adaptation to the running wheels, the rhythms of activity were individually analyzed over 10 consecutive days. The onset of activity was identified with a 5 min resolution and was defined as the first time point at which the mean intensity of activity was above 10 % of the maximum and remained above that point for at least 50 % of the time during the following 30 min. The reversed procedure was used for the cessation (offset) of activity (first time point below 10 % of maximum and activity remained below that point for at least 50 % of the time during the following 30 min). The time elapsing between the onset and offset of activity was defined as the total time of nocturnal activity, the peak value of activity and peak hour of activity were directly determined on the actogram for each animal. The mean 24h integrated activity was determined by adding 5 min -by- 5 min the mean number of revolutions in the wheel over 10 consecutive days for each animal. The data were then plotted with a 30 min resolution; this represented the mean distance run by the animals.

Jet lag

As chronobiological stressor, rats were subjected to an abrupt 6h advance shift in the LD cycle. On the day of the shift, lights were turned-off 6h before the current time, and the new 12h LD cycle (lights on: 0200h) was maintained thereafter. The time taken for re-entrainment to the new LD cycle was individually assessed using the "onset" of activity criterion

determined as previously. This criterion was defined as the smallest number of days required for the shifted activity onset to occur within 30 min of lights-off and to be stable for 3 days under the new LD cycle.

CRH radioimmunoassay

At the end of behavioral experiments, hypothalami from 7 months old rats, pair sibling with the animal of the running wheel experiment were rapidly dissected just after (within 5 min) the light switch on at 0800h and just after the light switch off at 2000h. To measure intrahypothalamic CRH, the hypothalami were snap-frozen and kept at −80°C until homogenization. The latter was performed in 1 ml of Tris-HCl 50 mM, pH 7.4, using a Teflon glass homogenizer. CRH was measured by radioimmunoassay (RIA) as previously described (Navarra et al., 1991), with the following modifications: a CRH antiserum (kindly donated by Pr. R. Bernardini) and a (2-[125I]-iodohistidyl32) CRF were used. The detection limit of the assay was 1 pg/tube (100 µl sample volume for incubation media), with intra and inter-assay coefficients of variation of 5 % and 10 %, respectively.

Statistical analysis

Data from the circadian running wheel experiment were analyzed using two-way analysis of variance [ANOVA; 2 groups (control and PRS) x 2 genders]. Data from the CRH hypothalamic content were analyzed using three-way analysis of variance [ANOVA; 2 groups (control and PRS) x 2 genders x 2 times (0800h and 2000h). The ANOVA analyses were always followed by Newman-Keul's *post-hoc* comparisons. The level of significance was set at $p<0.05$.

Results

Circadian rhythm of running wheel activity in male and female PRS rats

The circadian rhythms of running wheel activity under a regular 12/12 (0800h-2000h) light-dark cycle were individually analyzed in male and female control and PRS rats over 10 consecutive days of continuous registration (see Fig. 1). Male PRS rats anticipated the light switch off by starting the wheel running activity at 1907h when all the other groups started around 2000h, which is the precise time of light switch off (activity onset, ANOVA for group effect: $F_{(1;36)}=5.85$; $p < 0.05$ Newman-Keul's *post-hoc* test, Fig. 1, 2A). This phase advance of the activity onset in male PRS rats impacted also the activity offset, which was anticipated at 0631h whereas the activity offset in the other groups was around 0700h (activity offset, ANOVA for group x gender: $F_{(1;36)}=9.25$; $p < 0.005$ Newman-Keul's *post-hoc* test, Fig. 1, 2A). Duration of activity, calculated as the time between onset and offset and called "Alpha" (Fig. 2A), did not differ among the four groups.

Remarkably, there was a clear-cut gender effect of PRS on the levels of activity defined by the number of wheel revolution (Fig. 1, 2B,C).

First, during the period of lower activity, defined as the period between the offset of activity and the onset of activity of the next day, male PRS rats were more active than male controls with no difference between control and PRS female rats (ANOVA for group x gender: $F_{(1;36)}=7.68$; $p < 0.01$, Newman-Keul's *post-hoc* test, Fig. 2B). More interestingly, during the period of activity, defined as the period between the onset and the offset of activity, male PRS rats were again more active than male controls, whereas, in contrast, female PRS rats were less active than female controls (ANOVA for group x gender: $F_{(1;36)}=39.48$; $p < 0.00001$, Newman-Keul's *post-hoc* test, Fig. 2C). In addition, during this phase of activity control female rats were less active than control male rats (Newman-Keul's *post-hoc* test, Fig. 2C).

Six-hours phase advance jet lag

As an indicator of the effects of PRS on the ability to cope with a chronobiological stressor, we measured the number of days needed for the circadian rhythm of locomotor activity to become resynchronized after an abrupt 6h advance shift in the LD cycle (Fig. 3). Both PRS and gender influenced the time necessary for resynchronization (ANOVA for group x gender: $F_{(1;36)}=6.27$; $p < 0.05$, Fig. 4B). In control animals, females took more days than males to become resynchronized (Newman-Keul's *post-hoc* test, Fig. 3B). PRS increased the time to

become resynchronized to the new LD cycle in both genders, but to a larger extent in female than in male rats (Newman-Keul's *post-hoc* test, Fig. 3B).

Hypothalamic CRH levels at the beginning of the light and the dark phases

Hypothalamic CRH levels were measured at the beginning of the light phase at 0800h and at the beginning of the dark phase at 2000h (Fig. 4). Independently of the group or the gender, hypothalamic CRH levels were lower at 2000h than at 0800h (ANOVA for time effect, $F_{(1;40)}=39.81$, $p<0.000001$). This might reflect an increased CRH release at 2000h and an increased intracellular CRH accumulation due to a reduced release at 0800h.

The effect of PRS on hypothalamic CRH levels was gender dependent (ANOVA for group x gender: $F_{(1;40)}=34.27$, $p<0.00001$). In males, PRS increased CRH levels both at 0800h and 2000h (Newman-Keul's *post-hoc* test, Fig. 3). In females, PRS decreased CRH levels at 2000h (Newman-Keul's *post-hoc* test, Fig. 3) but did not cause changes in CRH levels at 0800h.

Discussion

Our results show a gender-specific outcome of PRS on circadian systems modulating locomotor activity, the resynchronization to the new light-dark cycle, and hypothalamic CRH levels. The pattern of locomotor activity in PRS rats was erratic and more fragmented, particularly in female PRS rats. However, total locomotor activity was increased in male PRS rats and decreased in female PRS rats compared to their respective controls. PRS caused a significant phase advance in the rhythm of circadian activity only in male rats. This is in agreement with our previous study showing that PRS induced a phase advance in the circadian rhythm of locomotor activity (Maccari et al., 2003). Furthermore, when subjected to an abrupt shift in the light-dark cycle, male and female PRS rats resynchronized their activity rhythm to the new light-dark cycle more slowly than control rats. Similar results were found using other models of early life stress. For example, male rats born from hypoxic mothers have showed significant alterations in the circadian rhythm of locomotor activity. They showed a phase advance of their rhythm of activity. After an abrupt 6h phase delay in the LD cycle, rats from the prenatal hypoxic group took significantly more time to resynchronize to the new LD cycle (Joseph et al., 2002). The altered circadian locomotor activity of PRS rats was associated with an increase in REM sleep previously showed in adult male rats (Dugovic et al., 1999; Mairesse et al., 2013).

These data suggest an involvement of the hypothalamic suprachiasmatic nuclei (SCN), which regulates the circadian clock in mammals (Moore and Eichler, 1972; Turek et al., 1995), and raise the possibility that the circadian clock is altered by early life stress. The SCN is composed of different sets of functionally distinct neurons. A particular set of SCN neurons has the function of conveying daily light-dark signals from other brain regions to “target” hypothalamic neurons. For example, serotonergic and cholinergic neurons regulate circadian locomotor activity *via* SCN neurons (Buijs and Kalsbeek, 2001). Both serotonergic and cholinergic neurotransmission might be involved in the effect of PRS on circadian rhythms. PRS impairs the development of serotonergic neurons (Peters, 1986), and PRS male rats show increased 5-HT1A mRNA levels in the cerebral cortex (Morley-Fletcher et al., 2004a). In addition, exposure to high glucocorticoid levels or acute stressors results into significant alterations in 5-HT turnover in the midbrain–pons area of PRS rats, which also show altered behavioral responses to 5-HT receptor agonists (Peters, 1988; Muneoka et al., 1997). During acute restraint stress, ACTH and CRH released under the influence of 5-HT might influence

REM sleep (Bonnet et al., 1997), which is abnormally increased in PRS rats (Dugovic et al., 1999; Mairesse et al., 2013).

The cholinergic system is involved in the executive mechanisms of REM sleep (Hobson et al., 1986), and PRS rats show a cholinergic hypersensitivity in response to a CRH challenge (Day et al., 1998). In addition, acetylcholine release in the medial prefrontal cortex and spontaneous locomotor activity are greater during the dark phase than during the light phase in rats of both sexes, and a positive correlation exists between acetylcholine release and spontaneous locomotor activity (Takase et al., 2009). Here, all groups of rats showed an increased locomotor activity during the dark phase rather than during the light phase. However, female PRS rats showed a lower locomotor activity and male PRS rats a greater locomotor activity than all other groups during the dark phase. We have shown previously that mild stress increases hippocampal acetylcholine release to a greater extent in PRS rats independently of the gender (Day et al., 1998). Thus, at least in the PRS model, acetylcholine release cannot be related to circadian motor activity, with the latter being highly gender-dependent in PRS rats. It will be necessary to measure biochemical and behavioral parameters in the same groups of control and PRS rats to establish whether changes in cholinergic or serotonergic transmission play any role in the gender-dependent abnormalities of circadian patterns in PRS rats.

In addition to serotonin or cholinergic systems, other factors may be involved in the long-term effects of PRS on circadian locomotor activity, such as CRH. CRH is involved in the regulation of physiological waking (Opp, 1995) and in sleep-wake modifications induced by acute stress exposure (González and Valatx, 1997). Under stress conditions, CRH acting as a neurotransmitter in the locus coeruleus induces an increased activity of noradrenergic neurons, which leads to an increase in paradoxical sleep (González et al., 1996; González and Valatx, 1997). The evidence that CRH neurotransmission is altered in PRS rats (Cratty et al., 1995) and that expression of CRH and its receptors is differentially affected by stress in male and female rats (Zohar and Weinstock, 2011) suggests a potential role for CRH in the regulation of circadian pattern in PRS rats. CRH regulates diurnal activity of the HPA axis, which, in the rat, peaks around the onset of the dark period (Girotti et al., 2007). The activity of CRH neurons within the hypothalamic paraventricular nucleus shows daily fluctuations and is controlled by the SCN (Carnes et al., 1990). At the beginning of the light phase, PRS caused a strong increase of hypothalamic CRH levels in males, but not in females. At the beginning of the dark phase, PRS increased hypothalamic CRH levels in males, but reduced

CRH levels in females. The increased CRH contents at the beginning of the light phase reflect a decreased release of CRH at this time of the day, when the activity of the HPA axis is low. CRH data at these 2 times of the day are in agreement with the locomotor hyperactivity of male PRS rats and the hypo-activity of female PRS rats during the dark period, and suggest a possible involvement of CRH in the regulation of circadian locomotor activity. Interestingly, it has been shown that the ratio of the transcripts of the two CRH receptors (CRH-R1 and CRH-R2) in the amygdala is increased in prenatally stressed males, but not in prenatally stressed females (Brunton et al., 2011). Male PRS rats show a strong anxiety component in their depression-like behavior (Morley-Fletcher et al., 2011), whereas female PRS rats show a depression-like behavior without an anxious component (Zuena et al., 2008; Van Waes et al., 2011). Thus, sex differences in anxiety-type behavior in PRS rats may be explained by the differential mRNA expression for CRH-R1, that is anxiogenic, and CRH-R2, that is anxiolytic, in the amygdaloid complex. The circadian variations of CRH found in PRS rats are in agreement with our previous data showing an altered circadian secretion of corticosterone plasma levels and hippocampal glucocorticoid receptors binding both in male and female PRS rats (Koehl et al., 1999).

Previous reports on long-term effects of PRS suggest that male PRS rats provide an animal model of depression associated with an anxiety-like phenotype (not seen in PRS females) and endowed with face, construct, and pharmacological validity (Maccari and Morley-Fletcher, 2007; Darnaudéry and Maccari, 2008; Zuena et al., 2008; Morley-Fletcher et al., 2011; Mairesse et al., 2013). Present data demonstrate that female PRS rats have abnormalities in the circadian locomotor activity, which are typical hallmarks of a depressive-like phenotype. This is consistent with the evidence that female PRS rats show an increased immobility in the forced swimming test that can be reversed by chronic alcohol administration (Van Waes et al., 2011). All together, these observations highlight the importance of the circadian system in the gender-specific outcome of PRS.

In conclusion, PRS induces an abnormal circadian function in adult rats as well as an increased response to stress (Darnaudéry and Maccari, 2008), suggesting an underlying dysfunction of their circadian clock and a global bad adaptation to challenges. One of the current hypothesis on the neuroendocrinology of depression involves a flattened (and advanced) circadian cortisol rhythm with hypercortisolism, possibly due to an increased sensitivity of the adrenal cortex (Holsboer et al., 1984) thought to normalize pituitary ACTH release in spite of an enhanced drive from the hypothalamic CRH neurons (Holsboer et al.,

1984; Gold et al., 1986). These hormonal features of depression can be related to those found in PRS rats. The persistence of all induced abnormalities after stressor removal could be seen as being particularly advantageous for the design and testing of new therapeutical strategies in circadian and stress-related disorders.

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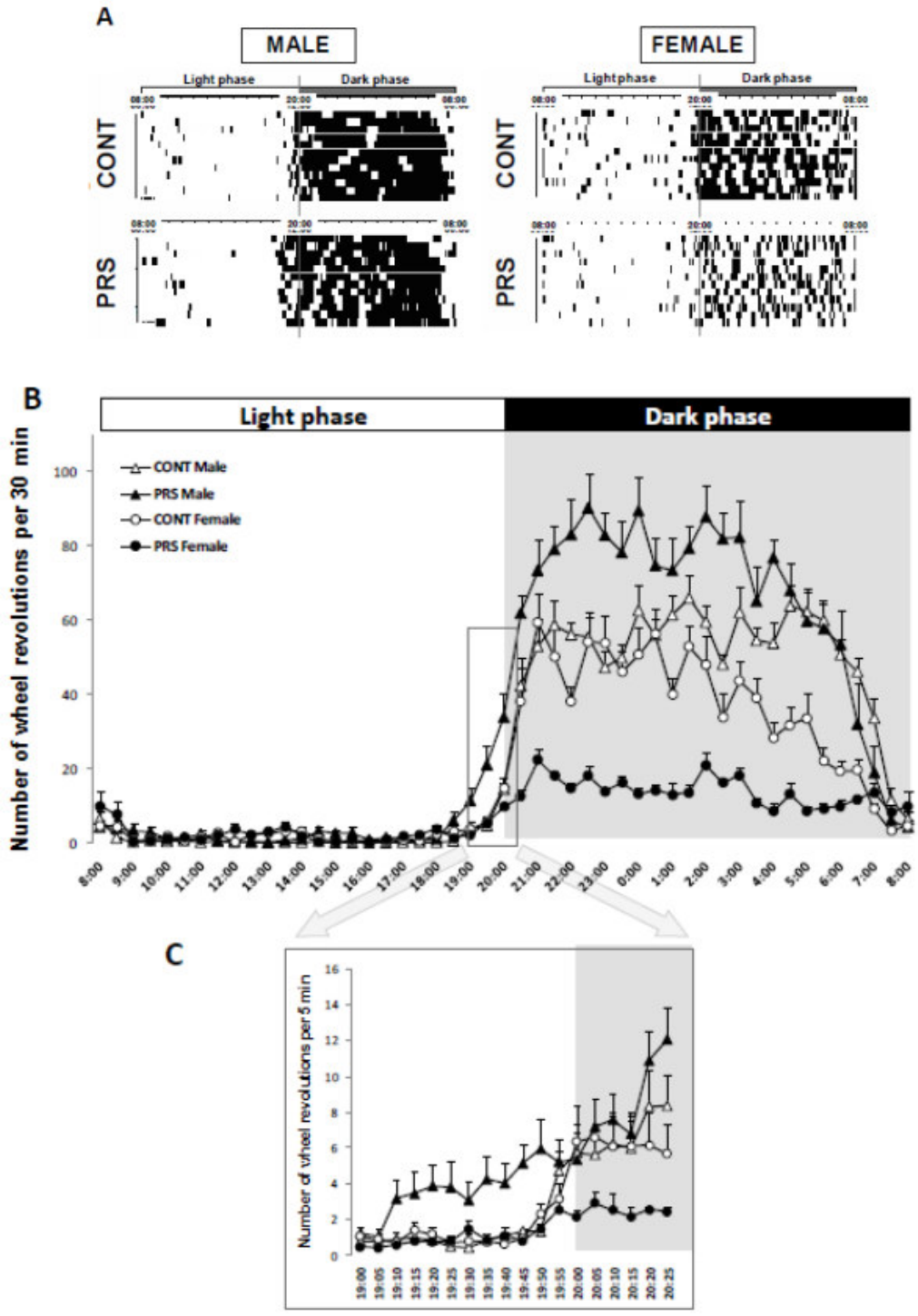


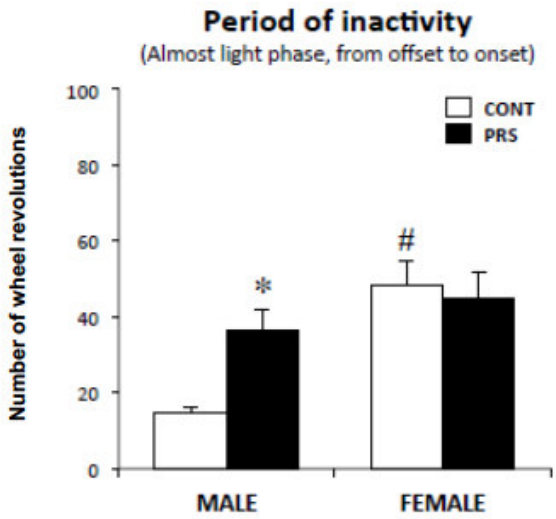
Figure 1. Van Camp et al.

A. Representative records of the circadian running wheel activity of male and female control and PRS rats over 12 consecutive days. The first LD cycle is shown at the top of each panel. Each horizontal line represents 24h of the animal's life composed from vertical bar, each representing a wheel revolution. B. The twenty-four hours representation of the mean number of wheel revolution calculated over 10 consecutive days and plotted with a 30 min resolution. C. Details of the light switch off period with data plotted with a 5 min resolution allowing a clear visualization of the activity phase advance in male PRS animals. Values are expressed as means ± S.E.M. n=10 rats per group.

A

	MALE			FEMALE		
	Control	PRS		Control	PRS	
Onset of activity	20h 01m 30s ±11m 24s	19h 07m 30s ±16m 55s	*	19h 50m 37s ±12m 56s	19h 38m 45s ±09m 25s	#
Offset of activity	07h 09m 00s ±13m 09s	06h 31m 15s ±09m 23s	*	06h 51m 15s ±03m 24s	07h 03m 45s ±04m 38s	#
Alpha (activity duration)	11h 09m 00s ±15m 06s	11h 23m 45s ±25m 26s		11h 00m 37s ±11m 51s	11h 25m 00s ±10m 21s	

B



C

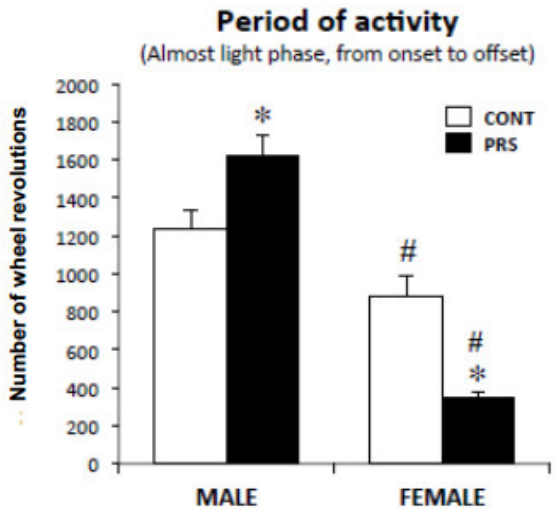


Figure 2. Van Camp et al.

A. Table for the onset, offset and alpha of running wheel activity considering the 0800h-2000h, 12/12 light-dark cycle. B. Total number of wheel revolutions during the period of inactivity corresponding to the period of time between the offset and the onset of activity, most of this period occured during light phase. C. Total number of wheel revolutions during the period of activity corresponding to the period of time between the onset and the offset of activity, most of this period occured during the dark phase. Values are expressed as means ± S.E.M. n=10 rats per group.* -$p<0.05$ PRS *vs*. control rats. # - $p<0.05$ female *vs*. male.

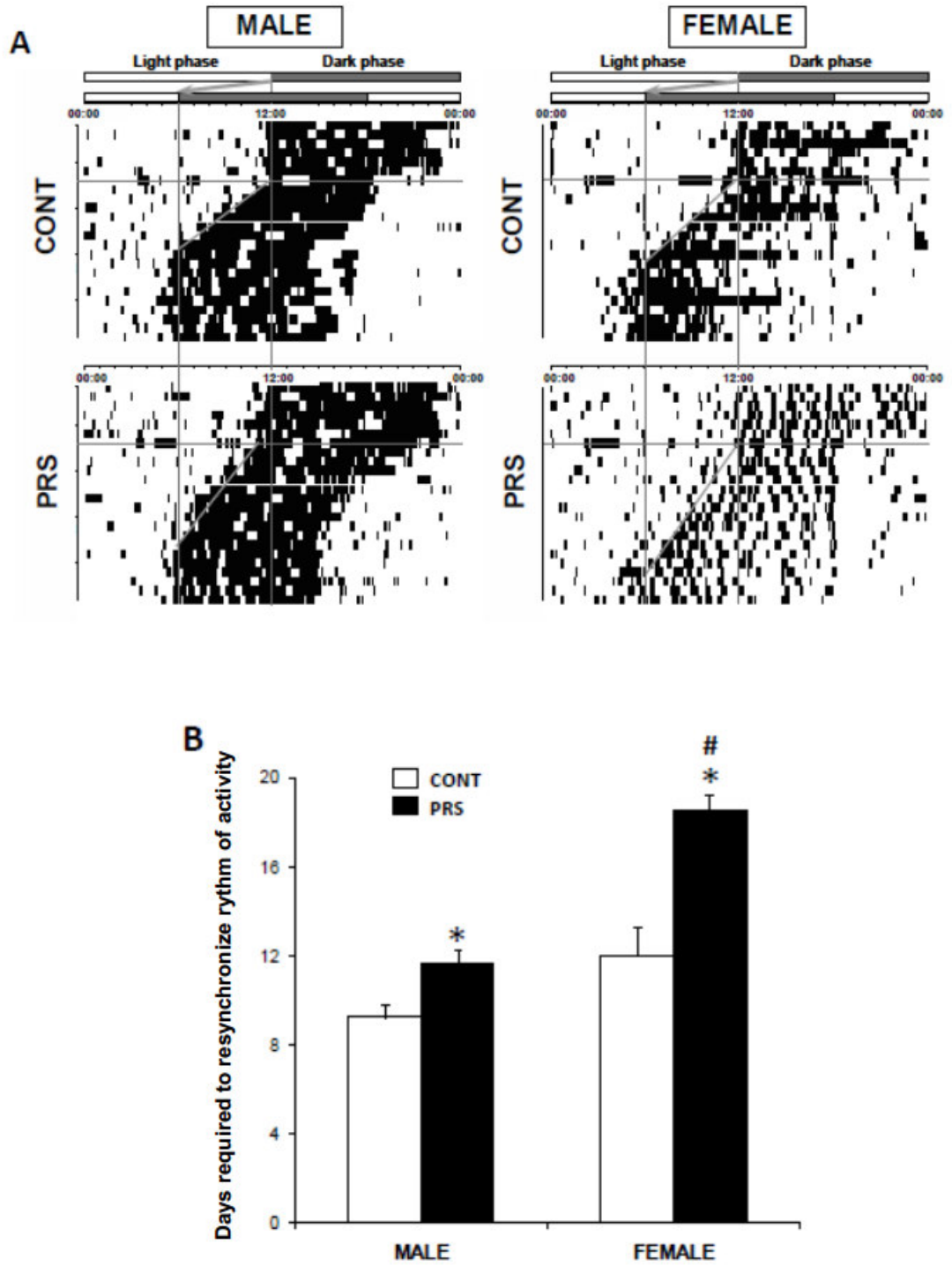


Figure 3. Van Camp et al.

A. Representative activity records of the circadian running wheel activity of male and female control and PRS rats subjected to abrupt 6h phase advance of the LD cycle (on day 7 on these records). The first LD cycle and the new LD cycle, after a 6h advances in the light are shown at the top of each panel. B. The time required by the animals to resynchronize their circadian rhythm of running wheel activity to the new LD cycle. Values are expressed as means ± S.E.M. n=10 rats per group.* -$p<0.05$ PRS *vs*. control rats. # - $p<0.05$ female *vs*. male.

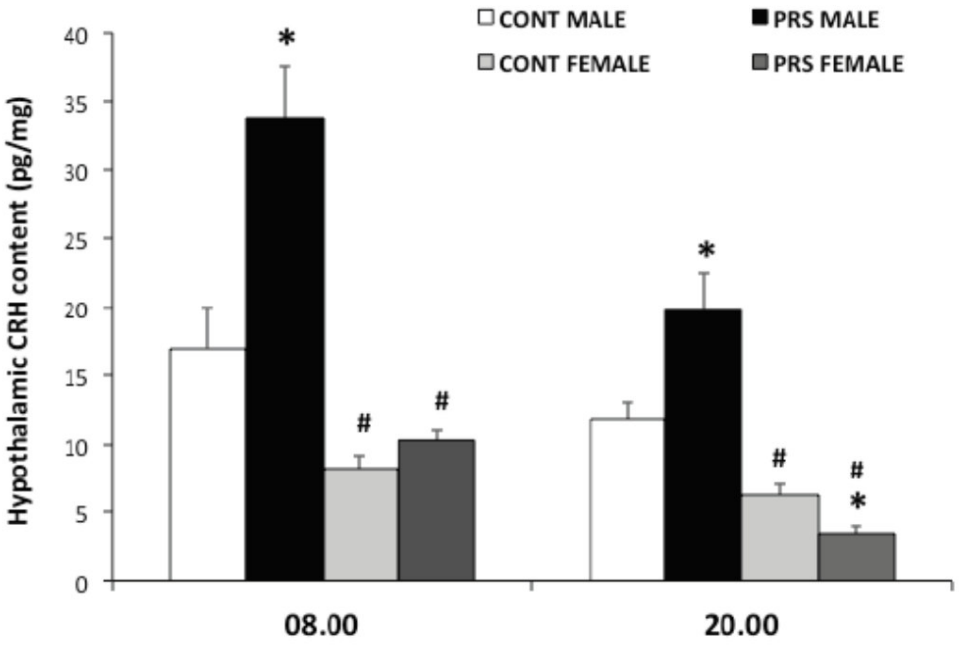


Figure 4. Van Camp et al.

Hypothalamic CRH content determined by I^{125} radioimmunoassay just after the light switch on at 0800h (A.) and just after the light switch off at 2000h (B.). Values are expressed in pg of CRH per mg of wet tissue as means ± S.E.M. n=6 rats per group.* -$p<0.05$ PRS *vs*. control rats. # - $p<0.05$ female *vs*. male.

HORMONAL MANIPULATION OF THE ANXIOUS /DEPRESSIVE PHENOTYPE

Hormonal fluctuations are very important in determining mood (reviewed by Van Wingen et al., 2011; Fernandez-Guasti et al., 2012). It is well established that across cycle, with fluctuations in estradiol and progesterone levels, women mood is extremely variable, with sometimes exacerbation of feelings of sadness, and depression (Schmidt et al., 1998; Bennett et al., 2004; Freeman et al., 2006), while testosterone levels in men appear positively associated with aggressive behavior and substance abuse (Dabbs and Morris, 1990), which are presumably mediated by its effect on social dominance (Tarter et al., 2007), and negatively associated with depressive mood (Kherea, 2013). During aging, an enhancement of anxious-/depressive like disorders is observed and estrogen and androgen supplementation therapies are efficient in the improvement of these troubles (Soares et al., 2001; Herrera-Pérez, Martínez-Mota and Fernández-Guasti, 2010)

In animal models, an antidepressant effect of testosterone was revealed in rats, both in the sucrose preference test, as a measure of anhedonia, and in the behavioral despair FST (Carrier and Kabbaj, 2012a).

Ovariectomy induces anxiety in female rats, which spend less time in the open arms of an EPM. One month treatment with daily subcutaneous injections with estradiol is able to improve this anxiety parameter (Nissen et al., 2012).

In PRS rats, an alteration of sexual patterns is observed, with a demasculinized profile in males (Ward, 1972; Weisz, 1983), even in second-generation offspring (Morgan and Bale, 2011). A decrease in aromatization process, as well as an increase in the 5α-reductase activity, responsible for the conversion of testosterone into its dihydrotestosterone metabolite was described (Reznikov et al., 2001). A testosterone injection at a very early stage (P1) was able to reverse the PRS-induced altered phenotype (Pereira, Bernardi and Gerardin, 2006). A decrease in testosterone and in estradiol levels, in male and female PRS rats was established, while PRS males displayed an increase in DHT levels, which was an important feature of the PRS-induced phenotype (Reynaert et al., submitted, Chapter 2). Thus, PRS females and males present sleep alterations and hormonal deficits that are comparable to the situation occurring during menopause and andropause.

As we have shown an important sex effect in PRS-induced anxious-/depressive-like disorders (Zuena et al., 2008; Weinstock, 2007; Morley-Fletcher et al., 2011; Van Waes et al., 2011),

confirmed in the present chapter with the circadian pattern of locomotor activity, we wanted to determine how sex hormones could influence mood disorders in the PRS model.

At adulthood, rats were submitted to the following procedures: sham-operation, ovariectomy or hormonally supplemented with silastic tubes filled with dihydrotestosterone (DHT), finasteride (FIN) or estradiol (E). On month after surgeries, for recovery and hormonal statement, rats were tested for their anxious-/depressive-like behavior, respectively in the elevated plus maze apparatus (Fig. 16) and the splash test (Fig. 17).

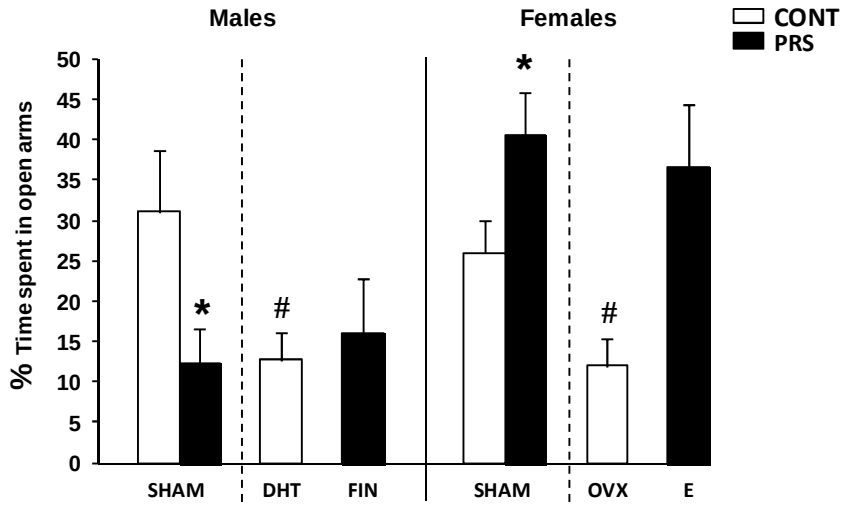
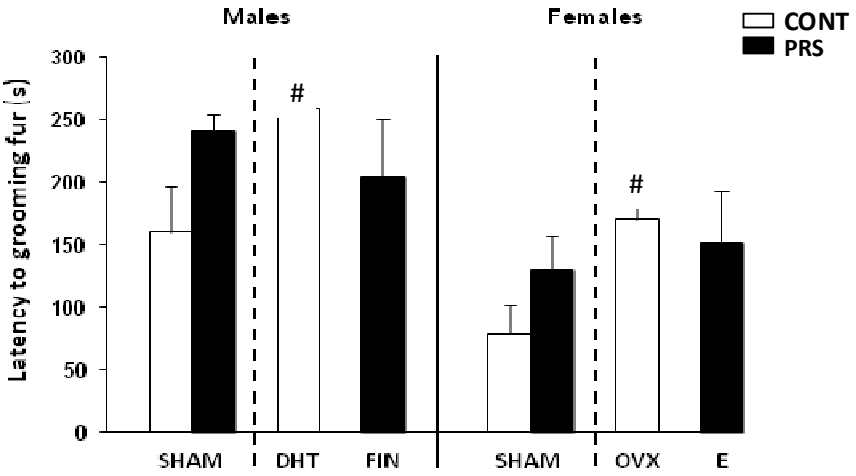


Fig 16- Gonadal hormones modulation of anxious-like phenotype in Control and PRS male and female rats.

One month after surgeries and hormonal treatment, rats (n=7-8) per group were tested in the Elevated Plus Maze apparatus. Experiments were performed between 2:00 and 5:00 PM. Results are represented as the mean ± S.E.M of the percentage of time spent in the open arms. SHAM=sham-operated animals, DHT=dihydrotestosterone supplemented, FIN=finasteride-supplemented, OVX=ovariectomy, E=estradiol-supplemented. *p<0.05 vs. Control animals of the same sex, #p<0.05 vs SHAM animals of the same group (CONT or PRS).



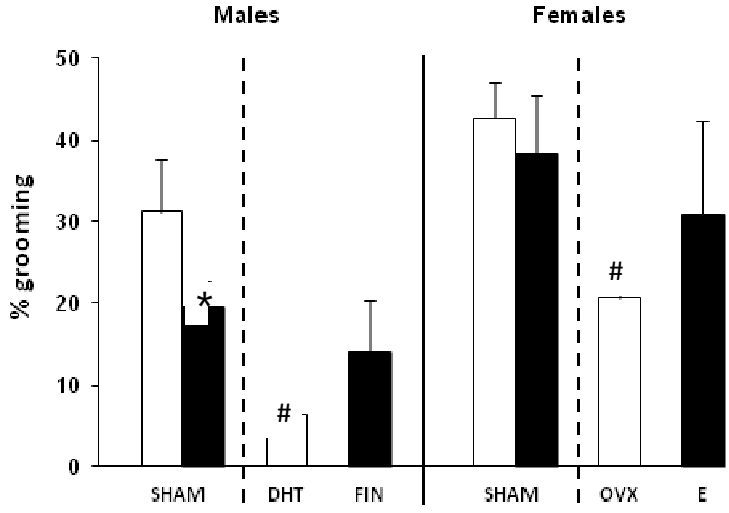


Fig 17- Gonadal hormones modulation of depressive-like phenotype in Control and PRS male and female rats.

One month after surgeries and hormonal treatment, rats (n=7-8) per group were tested in the Splash test. Experiments were performed between 2:00 and 5:00 PM. Results are represented as the mean ± S.E.M of the latency to groom fur (s) (A) and of the percentage of time spent in grooming (B). SHAM=sham-operated animals, DHT=dihydrotestosterone supplemented, FIN=finasteride-supplemented, OVX=ovariectomy, E=estradiol-supplemented. *$p<0.05$ vs. Control animals of the same sex, #$p<0.05$ vs SHAM animals of the same group (CONT or PRS).

CHAPTER II: PRS, SEX DIFFERENCES AND SEX HORMONES ROLE ON PREFERENCE FOR REWARDING STIMULUS

3- Locomotor activity response to cocaine is predictive for drug-induced CPP: sex and stress as modulators

In the previous chapter, we have shown the importance of considering sex differences in the features obtained in the PRS model, and given evidence for a role of sex hormones in mediating PRS-induced anxious-like and/or depressive-like symptoms.

Here, we addressed the question of sex differences in the PRS effect on vulnerability to the psychostimulant drug cocaine, considering both locomotor-activating effect of the drug, and its ability to induce a conditioned place preference. In a context where some responses are predictive of drug self-administration (locomotion, Piazza et al., 1989; novelty seeking, Klebaur and Bardo, 1999), we wondered if an increase in locomotor activity could be predictive of an enhanced sensitiveness to cocaine-induced preference, as already revealed for amphetamine (Mathews, Morrissey and McCormick, 2010).

Manuscript in preparation

Locomotor activity response to cocaine is predictive for drug-induced conditioned place preference in male and female rats

Marie-Line Reynaert[1,4], Eleonora Gatta[1,4], Jordan Marrocco[2], Jérôme Mairesse[1,4], Gilles Van Camp[1,4], Ferdinando Nicoletti[3,4], Stefania Maccari[1,4*], Sara Morley-Fletcher[1,4*]

[*]co-last authors

[1]Neural Plasticity Team, UMR 8576/UGSF, CNRS/University Lille1, Lille, France
[2]IRCCS Centro Neurolesi "Bonino-Pulejo", Messina, Italy
[3]Dept of Pharmacology, Sapienza University of Rome and IRCCS Neuromed, Pozzilli, Italy
[4]LIA - International Associated Laboratory – Prenatal Stress and Neurodegenerative Diseases, University of Lille1/CNRS, Villeneuve d'Ascq, France; Neuromed, Pozzili, Italy; Sapienza University of Rome, Italy.

Address for correspondance:
Prof. Stefania Maccari, Ph.D., HDR
University Lille 1, France,
Co-Director LIA
Neural Plasticity Team – CNRS UMR 8576/ UGSF
Structural and Functional Glycobiology Unit
Bât C9, Avenue Mendeleiev –59655 Villeneuve d'Ascq France
Office: +33.32033.6042; Fax +33.32043.6555
e-mail : stefania.maccari@univ-lille1.fr

Abstract (240 words/250)

Behavioral sensitization to psychostimulant drugs is known to highly contribute to drug addiction. Here we found that adult rats of both sexes subjected to prenatal restraint stress ("PRS rats") develop locomotor sensitization to escalating doses of cocaine under conditions in which sensitization did not develop in age- and sex-matched unstressed rats. PRS rats also showed a greater cocaine-preference in a conditioned place preference (CPP) paradigm. Locomotor response to cocaine was highly correlated to cocaine preference in both sexes. PRS rats show abnormalities in gonadal hormones, characterized by higher levels of dihydrotestosterone (DHT) in males and lower levels of 17-β-estradiol (E_2) in females. To examine where these changes were causally related to the increased sensitivity to cocaine, we assessed cocaine-induced CPP in unstressed and PRS rats after hormonal manipulations aimed at modifying DHT and E_2 levels in males and females, respectively. Supplementation of DHT in male unstressed rats enhanced CPP to the same levels observed in PRS rats, whereas inhibition of DHT synthesis with the 5α-reductase inhibitor, finasteride, reduced CPP in male PRS rats. In contrast, E_2 supplementation failed to reduce CPP in female PRS rats, although ovariectomy increased CPP in female unstressed controls, as expected. These findings suggest that early life stress interacts with gender in shaping the vulnerability to drug addiction, and that, in males, inhibitors of 5α-reductase are of potential value in restraining drug abuse in individual with a positive anamnesis of stress in the perinatal life.

Keywords: conditioned place preference, cocaine, gender, sex hormones, prenatal stress

Introduction

Sex is an important basic human variable; however, women continue to be underrepresented in clinical trials. As such, women remain a vulnerable population subject to the adverse effects of pharmacological therapies. Unfortunately, selection of male animals is often the "default" choice, and additional information related to sex in preclinical testing should be considered when designing and analyzing studies in all areas and at all levels of biomedical and health-related research (Raz and Miller, 2012; Zucker and Beery, 2010; Beery and Zucker, 2011).

Stressful life experiences, such as maltreatment, catastrophic events, occupational or financial difficulties are known as important factor in the etiology of drug addiction and lead to an enhanced risk of developing alcohol abuse (reviewed by Keyes et al., 2011). Several preclinical studies have shown the ability of stressors to alter the acquisition of drug self-administration in rats (Goeders, 2002; Piazza and Le Moal, 1998). Procedures aiming at decreasing corticosterone levels, such as adrenalectomy or injection of glucocorticoids synthesis inhibitor, metyrapone, decrease the sensitiveness of animals to the addictive properties of drugs (Goeders and Guerin, 1996; Rougé-Pont et al., 1995; Marinelli et al., 1997).

The model of prenatal restraint stress (PRS) in rats is a well-documented model of dysregulation of HPA axis characterized by a prolonged secretion of corticosterone in response to stress (Koehl et al., 1997, 1999; Dugovic et al., 1999) which results from a reduced expression of type I and - II corticosteroid receptors in the hippocampus (Maccari et al., 1995). An impairment in corticosteroids negative feedback associated with higher corticosterone levels is also found in rats predisposed to self-administer amphetamine (Maccari et al., 1991; Piazza et al., 1991). Remarkably, PRS rats display an enhanced propensity to amphetamine or cocaine self-administration (Deminière et al., 1992; Kippin et al., 2008), an increased locomotor response to nicotine and amphetamine (Koehl et al., 2000; Henry et al., 1995), as well as a reduced metabolism of 3,4-methylendioxymethamphetamine (ecstasy) (Morley-Fletcher et al., 2004).

It has also been demonstrated that the locomotor response to psychostimulants may predict consumption of the drug (Piazza et al., 1989; De Wit and Phillips, 2012). For example, a greater sensitivity to locomotor effects of the drug was associated with higher levels of self-administration (Deminière et al., 1989; Piazza et al., 1989). This relationship has also been shown for cocaine both in male and female rats (Mantsch et al., 2001; Zhao and Becker, 2010).

Of note, sex differences are also important in drug addiction and, women are more sensitive to the effects of drugs of abuse although they consume less. Once addicted to a drug, women find

more difficult to stop consumption than men do, and, after an abstinence period, women are more vulnerable to relapse (Becker and Hu, 2007; Fox and Sinha, 2009). A quicker transition to addiction is also observed in animal models. Accordingly, female rats exhibit higher response to conditioned place preference (CPP) for cocaine and require less pairing sessions to develop cocaine-CPP, with lower doses than males (Russo et al., 2003). Female rats also display an increase in acquisition of drug self-administration , with a high breaking point in comparison to males, which reflects the motivation of the animal to catch the drug (Kerstetter and Kippin, 2011; Hu et al., 2004; Lynch et al., 2008). The greater sensitivity of females to drugs of abuse is mediated by estradiol (E_2), because E_2 administration enhances both drug-seeking behavior and sensitization to cocaine, and ovariectomy decreases motivation to drug intake (Lynch, 2006; Hu *et al*., 2004; Segarra, 2010; Jackson et al., 2006). In addition, drug craving and reinstatement of drug seeking are more prominent during the estrus phase of the follicular cycle (Kippin et al., 2005). E_2 enhances the activity of the brain-reward system in rats (Galankin et al., 2010), and administration of tamoxifen, a selective estrogen receptor modulator, decreases morphine-induced CPP in mice (Esmaeili, 2009).

In males, the role played by androgens in the vulnerability to drug addiction is controversial. Of note, most of the findings relied on the effect of castration and testosterone (T) supplementation or replacement (Camp and Robinson, 1988; Van Luijtelaar et al., 1996; Walker et al., 2001; Chin et al., 2002; Russo et al., 2003; Minerly et al., 2008;), and, to our knowledge, there are no studies that specifically examine the role played by dihydrotestosterone (DHT), the major androgenic metabolite of T, in drug addiction.

It is important to examine the effect of gender and sex steroids on drug abuse taking into account the potential impact of early life stress. PRS rats offer a valuable model for the study of the interaction among early life stress, geneder, sex steroids, and drug abuse. Some (but not all) biochemical and behavioural phenotypes of PRS rats are gender-dependent (Zuena et al., 2008). In addition, prenatal stress causes long-lasting changes in sex steroids in both sexes, which include *inter alia* reductions of T levels and increases in DHT levels in males, and reductions in E_2 levels in females (Ward, 1972; Weisz et al., 1982; Murase, 1994; Reznikov *et al*., 2001; Reznikov and Tarasenko, 2007; Reynaert, SFN, 2012). We have found recently that changes in DHT in males and in E_2 in females are causally related to the abnormalities in hedonic sensitivity to natural rewards displayed by male and female PRS rats, respectively (Reynart et al., SFN, 2012). Here, we examined how gender/sex steroids and early life stress influence the vulnerability to psychostimulants by assessing locomotor response to cocaine and cocaine-dependent CPP in unstressed and PRS rats.

Methods

Animals

Adult female Sprague-Dawley rats weighing about 250 g and sexually-experienced males (400-500 g) were purchased from Charles River Laboratories (L'Arbresle Cedex FRANCE). Females were group-housed for 3 weeks for acclimation and oestrous cycle coordination in a temperature (22+/-2°C) and humidity-controlled room under a 12h light-dark cycle with lights off at 20h. Males were single-housed during this period. Water and chow were provided *ad libitum*. Then, females were placed with a male for a night and the day corresponding to spermatozoids revealing by microscopy or copulation plug visualisation was designated as embryonic day 0 (E0), and females were housed individually in transparent Plexiglas cages and randomly assigned to control or stressed group (n=14 per group).

Prenatal restraint stress procedure

Pregnant females were subjected to restraint stress according to our standard protocol (Maccari et al., 1995). At E11 of pregnancy until delivery, female rats were submitted to three stress sessions daily (45 min each), during which they were placed in transparent plastic cylinders and exposed to bright light or were left undisturbed (control dams).

Only rats from litters of 10-14 rats with a similar number of males and females were used. After weaning (P21), offspring grew up (2-3 brothers/2-3 sisters per cage) until reaching the good stage (adulthood) for experimentation. A maximum of two animals was used for a same group to avoid any litter effect (Becker and Kowall, 1977; Chapman and Stern, 1979). All experiments followed the rules of the European Communities Council Directive 86/609/EEC. The local ethics committee approved the prenatal stress procedure. Separate sets of adult rats (3-4 months of age) were used for surgeries and/or behavioural and hormonal analyses.

Drug administration

Cocaine (cocaine hydrochloride, Sigma-Aldrich, France) was prepared extemponaneously in saline solution (NaCl 0.9%) and administered intraperitoneally (i.p.). For chronic administration of cocaine, escalating doses of the drug were administered for 6 days (15 mg/mL/kg for 2 days, 20 mg/mL/kg for 3 days and 30 mg/mL/kg on day 6).

Locomotor activity

The impact of PRS and sex on cocaine locomotor-activating effect was assessed on day 1 (acute effect, single injection of 15 mg/mL/kg) and day 6 of the chronic administration protocol (30 mg/mL/kg). Control and PRS male and female adult rats were placed in locomotor activity cages for 15 min for habituating and then, were injected with vehicle or cocaine, and replaced in the cages of the locomotor activity apparatus, for 90 min. The activity in the front and the back of the cage, as well as rearing and moving forwards and backwards were automatically recorded (i-metronic, Pessac, France). Results are represented as the sum of all the locomotor activity parameters and as 10 min interval at the 90 min recording session.

Conditioned Place Preference (CPP)

Saline solution (NaCl 0.9% in distilled water) was used as vehicle neutral stimulus, whereas cocaine (15 mg/mL NaCl 0.9%/kg) was used as rewarding stimulus. To evaluate the influence of PRS and sex differences on preference to cocaine-paired chamber, adult (4 mo old) male and female control and PRS rats (n=7-8 rats per group) were used for the experiment. The CPP apparatus was made in opaque Plexiglas and had two compartments with different associated visual cues (one chamber white and the other grey). On day 1 (pretest), rats were allowed to explore the whole apparatus for 20 minutes in the absence of any stimulus, in order to determine their spontaneous preference for one chamber of the apparatus. Conditioning (8 days, 30 min/session) with cocaine was conducted in the least preferred side while vehicle injection was paired with preferred side, as determined after pretest.

During conditioning sessions, animals were alternatively injected with vehicle or cocaine just before being placed in the appropriate stimulus-paired chamber. Day 10 (test) was performed as pretest. The time spent in vehicle- and cocaine- paired chambers was automatically recorded (i-metronic, Pessac, France). Data were expressed as a percentage of time spent in cocaine-paired chamber during the test minus the pretest.

In a second time, we addressed the question of the link between cocaine CPP and response to cocaine-induced locomotor activity. To address this issue, 24 h after CPP test, rats underwent the chronic administration protocol, as explained above. We have also addressed the question of sex hormones in modulating cocaine-induced CPP.

Surgical Procedures

To assess the effect of sex hormones on rats preference for cocaine, many hormonal modulations were carried out. Male and female rats (6-9 per group) were anesthetized with 1 mL/kg of a solution of ketamine hydrochloride (100 mg/kg IP), xylazine (8 mg/kg IP) and acepromazine (1 mg/kg IP), and one of the following procedures was performed (ovariectomy (OVX), sham ovariectomy (SO). Ovariectomies consisted in bilateral dorsal incisions (1.0-1.5 cm), 2 cm below the last rib. After removal of the gonads, the remaining tissue was replaced into the peritoneal cavity and the skin incision closed with suture. Betadine was applied to ensure the appropriate asepsis and antisepsis. Sham surgeries were carried out anesthetizing the animals and making incisions, but not removing gonads.

For the hormonal study, 10 mm silastic tube implants (inner diameter 1,98mm; outer diameter 3.18 mm; Biesterfeld France, Rueil-Malmaison Cedex, France) were placed subcutaneously in the midscapular region during surgery. Empty implants were used as control in gonadectomized and SO rats, whereas rats with hormonal supplementation were sham-operated and implanted with capsules filled either with dihydrotestosterone (DHT), estradiol benzoate (E_2) or finasteride (FIN). All products were provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Implants were expected to supply the hormones for two months at a level close to the physiological one (Van Coppenolle et al., 2001).

One month after surgeries, rats belonging to the various experimental groups were tested for cocaine preference in the CPP paradigm, as aforementioned.

Statistical analysis.

Data were analyzed by two-way ANOVA for locomotor activity (group by sex), three-way ANOVA (group by sex by treatment) for CPP test or one way ANOVA for CPP test after hormonal manipulation. The Fisher's post hoc test was used to isolate the differences. Correlation was analyzed using the Pearson's correlation analysis A p value <0.05 was considered to be statistically significant.

Results

Gender and early-life stress influence the locomotor response to cocaine.

We studied the locomotor response of PRS and unstressed male and female rats to the a single or repeated injections of cocaine (escalating doses from 15 to 30 mg/kg in6 days).

A single injection of cocaine (15 mg/kg) increased locomotor activity to the same extent in unstressed and PRS rats. However, the locomotor response was much greater in females than in males. No significant effect of PRS was seen (ANOVA, sex x treatment effect $F_{(1,62)}$=23.0, p<0.001, **Fig. 1A**).

After 6 days of repeated injections, the locomotor activity response remained stable in unstressed rats of both sexes. In contrast, PRS rats showed an enhanced response to cocaine after 6 days of treatment. An higher response in female rats was still observed (ANOVA, group x treatment effect, $F_{(1,58)}$=10.50, p<0.01, **Fig. 1B**).

Figures 2C to F show locomotor activity recorded at 10 min intervals during the 90 min sessions in response to single or repeated cocaine injections.

After 6 days of cocaine injections, the locomotor response to cocaine shows a U-shaped curve as a function of the observation, time. PRS males showed a reinforced response to cocaine at all times, as compared to unstressed control males.

In contrast, female PRS rats showed a great locomotor response as compared to unstressed females only after the first 10 min of observation. We wish to highlight that in females, locomotor sensitization could be seen in the first 10 min of observation, when total locomotor activity was increased by 50% in PRS rats and by about 2-fold in unstressed rats, as compared to values observed after an acute injection (**compare Fig. 1E with 1F**).

PRS enhanced preference for cocaine in both male and female rats.

We examined the conditioned place preference for cocaine in PRS and unstressed male and female rats. We found an enhanced preference for cocaine-paired chamber in PRS rats of both sexes. As already observed for locomotor activity, place preference was substantially greater in females than in males in both experimental groups (ANOVA, group effect, $F_{(1,28)}$=9.02, p<0.01, sex effect, $F_{(1,28)}$=10.03, p<0.01, **Fig. 2**).

Increase in cocaine CPP is correlated with increase in cocaine-induced locomotor activity.

To examine whether responses to cocaine in the two behavioral paradigms were correlated, we designed a experiment in which the same animals underwent conditioned place preference for 8 days (4 days of pairing with cocaine or saline) and, 48 hours later, 6 days of escalating doses of cocaine (see above), followed by measurement of locomotor activity. We showed a positive correlation between the percentage of time spent in the cocaine–paired chamber and the total locomotor activity (Pearson's correlation coefficient, $r= 0.40$, $p<0.01$, **Fig. 3**).

Modulation of cocaine preference by hormonal manipulation in relation to gender and early life stress.

We moved from the evidence that DHT levels are higher in PRS rats as a result of an increase activity of 5α-reductase (the enzyme that converts T into DHT) (Reznikov and Tarasenko, 2007). In contrast, E2 levels are lower in female PRS rats as compared to unstressed controls. Manipulations that restore hormonal balance correct abnormalities in hedonic sensitiveness to natural reward in PRS rats (Reynaert et al., submitted). We examine whether changes in DHT levels in males and E2 levels in females could be responsible for the reinforced response to cocaine in PRS rats, we use the following strategy: (i) male PRS rats were treated with the 5α-reductase inhibitor finasteride (subcutaneous 1 cm silastic tube filled with finasteride), (ii) male unstressed rats were supplemented with exogenous DHT (subcutaneous 1 cm silastic tube filled with DHT), (iii) female PRS rats were supplemented with E2 (subcutaneous 1 cm silastic tube filled with E2), (iv) female unstressed rats were ovariectomized. Data in males were fully consistent with a positive role for DHT in shaping cocaine preference. Accordingly, unstressed male rats supplemented with DHT displayed a cocaine preference similar to that displayed by PRS rats, and finasteride treatment in PRS rats lowered cocaine preference to the same levels obtained in unstressed rats. Data in females were less clear, with ovariectomy increasing cocaine preference in unstressed controls but estradiol supplementation failing to lower cocaine preference in PRS rats (ANOVA, $F_{(5,57)}=3.94$, $p<0.01$, **Fig . 4**).

Discussion

We have shown that unstressed and PRS female rats displayed a higher response to cocaine-induced locomotor activity, and an increase in conditioned place preference (CPP) for cocaine as compared to the respective groups of male rats. This is consistent with a large body of evidence indicating a greater sensitivity of female rats to cocaine, in terms of locomotor response, CPP, self-administration, and drug-reinforced operant behavior (Chin et al., 2002; Festa et al., 2004; Sell et al., 2000, Van Haaren and Meyer, 1991; Walker et al., 2001; Becker et al., 1982; Harrod et al., 2005; Kantak et al., 2007; Lynch and Carroll, 1999; Lynch and Taylor, 2004; Roth and Carroll, 2004; Russo et al., 2003; Walker et al., 2001).

At least under our conditions, escalating doses of cocaine did not produce locomotor sensitization in unstressed rats. Interestingly, however, PRS rats showed a greater locomotor response to the sixth injection of cocaine with respect to the first injection, indicating that sensitization developed only in animals exposed to early life stress. This suggests that early life stress causes neuroadaptive changes in the mesolimbic system that progressively reinforce the action of cocaine. So far, an enhanced behavioral sensitization to psychostimulant drugs has been found only in male PRS rats (Deminière et al., 1992; Koehl et al., 2000, Kippin et al., 2008). Here, in contrast, both males and female PRS rats showed an enhanced motor response to repeated injections of cocaine, with females maintaining a greater response than males. Similarly, both male and female PRS rats showed a greater cocaine preference in the CPP paradigm, indicating that the higher vulnerability to psychostimulants of PRS rats is not a prerogative of the male gender. We found a positive correlation between locomotor response and cocaine preference, in agreement with the evidence that rats with greater behavioral sensitization have a higher vulnerability to drug addiction (Piazza et al., 1989, Zhao and Becker, 2010).

Of note, both male and female PRS rats display depressive-like behavior, whereas other behavioral phenotypes of PRS rats show a gender effect (Morley-Fletcher et al., 2003, Zuena et al. 2008; Van Waes et al., 2011). This raises the interesting possibility that, in PRS rats, the greater preference for cocaine is related to the depression-like behavior, perhaps as a form of self-medication.

Sex hormones shape the response to psychostimulant drugs and are important in determining sex differences in drug addiction (Lynch, 2002). 17-β-Estradiol (E_2) has been implicated in the greater response of females to cocaine (Segarra et al., 2010; Russo et al., 2003), perhaps as a result of its action on the dopaminergic system (Walker et al., 2012).

The role of T and its metabolite, DHT, in males, on mechanisms of reward and drug addiction, is less established (Festa and Quinones-Jenab, 2004). In a previous work, we have shown that the effect of early life stress on hedonic sensitivity to natural rewards in males and females were mediated by DHT and E_2, respectively (Reynaert et al., SFN, 2012). In particular, male PRS show higher levels of DHT and female PRS rats lower E_2 levels as compared to sex-matched unstressed rats. Inhibition of DHT synthesis with finasteride in male rats and E_2 supplementation in female rats correct the abnormalities in hedonic sensitivity for natural rewards in PRS rats (Reynaert et al., SFN 2012). Here, we found a similar scenario in male rats, where treatment of unstressed controls with exogenous DHT enhanced cocaine preference to the same levels found in PRS rats, whereas treatment of PRS rats with finasteride reduced cocaine preference. In contrast, E_2 supplementation failed to affect cocaine preference in female PRS rats, although ovariectomy increased preference in unstressed rats (see also Bobzean et al., 2010). A comparison between present data on cocaine preference and our previous data on sensitivity to palatable food (Reynaert et al., SFN, 2012) suggests that the response of the reward circuit to natural rewards and psychostimulant undergoes the same regulation by early life stress and sex steroids in male but not in female rats. Perhaps this reflects a greater addictive property of food in males, which, at least in some animal species, can be evolutionistically related to the predominant food-seeking behavior of the male gender.

The role of DHT in cocaine preference that emerges from the comparison between unstressed and PRS rats is consistent with the view that androgens regulate the vulnerability to drug addiction (Menéndez-Delmestre and Segarra, 2011, but see also Minerly et al., 2008 for a contrasting view). Of note, anabolic androgen steroids (AAS) abusers have a greater tendency to become addicted to other drugs of abuse, such as cocaine, heroin, amphetamine, and 3,4-methylenedioxymethamphetamine (DuRant et al. 1995; Kindlundh et al., 2001; Thevis et al. 2008; Hakansson et al., 2012). In addition, AASs induce long-lasting changes in the rat brain reward circuits associated with drug dependence (Kailanto et al., 2011).

In conclusion, we have found that early life stress and gender interact in shaping the response to, and preference for, cocaine in rats. The role played by DHT in the greater cocaine preference of PRS rats suggests a potential use of 5α-reductase inhibitors (e.g., finasteride and dutasteride) to restrain drug addiction in people with a positive anamnesis of early life stress.

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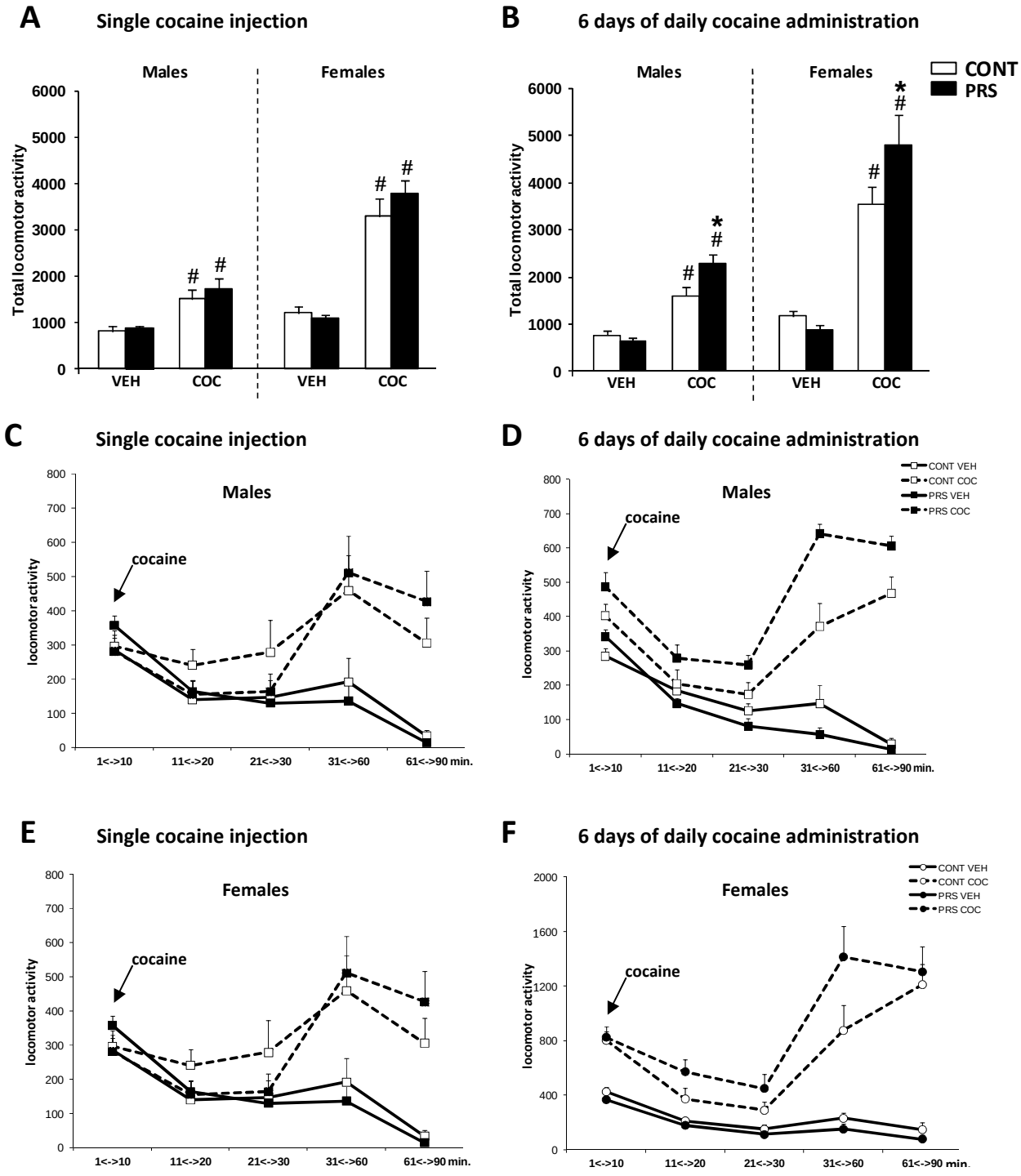

Fig. 1- Females and PRS rats are more sensible to behavioral sensitization with cocaine.

On day one of the chronic sensitization procedure, male and female control unstressed and PRS rats are tested for their sensitiveness to the effect of an acute injection of cocaine (15 mg/mL/kg, i.p.) in comparison to rats treated with vehicle (A). On day 6, rats are injected with cocaine (30 mg/mL/kg, i.p.) or vehicle and tested for their locomotor activity response (B). Data are expressed both as total locomotor activity during the 90 of test and as a kinetic. Values are means ± SEM of 7-8 rats per group, *p<0.05 vs control animals, # p<0.05 vs vehicle-treated animals of the same group (Control or PRS) and sex. CONT=control unstressed rats, VEH=vehicle-treated, COC=cocaine-treated.

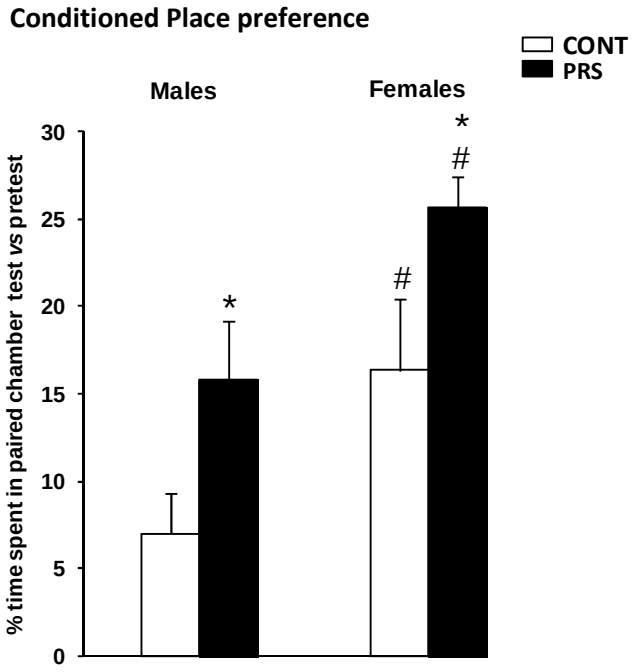

Fig. 2- Females and PRS rats are more sensible to the reinforcing effect of cocaine in a Conditioned Place Preference paradigm.

The percentage of time spent in the cocaine-paired chamber during the test minus the pretest is represented. Values are means ± SEM of 7-8 rats per group, *$p<0.05$ vs control animals of the same sex, # $p<0.05$ vs males of the same group (Control or PRS) and sex. CONT=control unstressed rats.

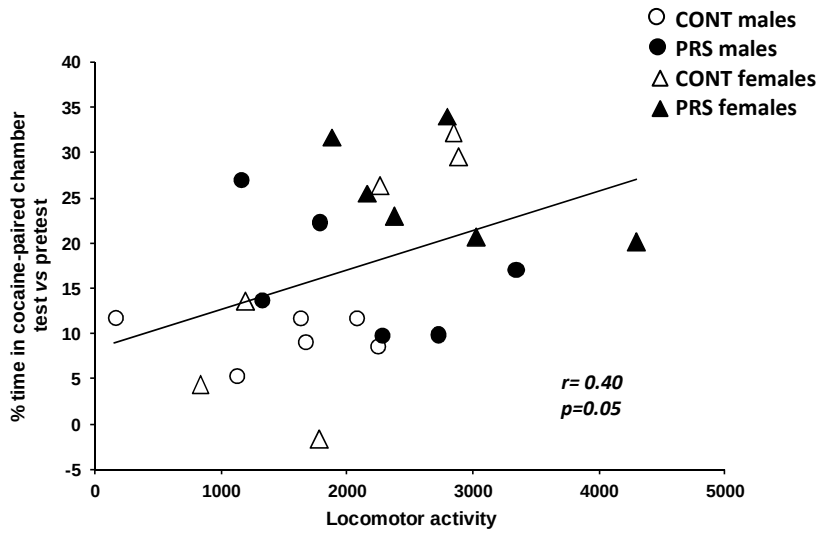

Fig. 3- Correlation analysis of cocaine-induced Conditioned Place Preference toward cocaine-induced locomotor activity.

Pearson's correlation coefficient (r) and related p value is reported in the figure (n=6 rats per group).

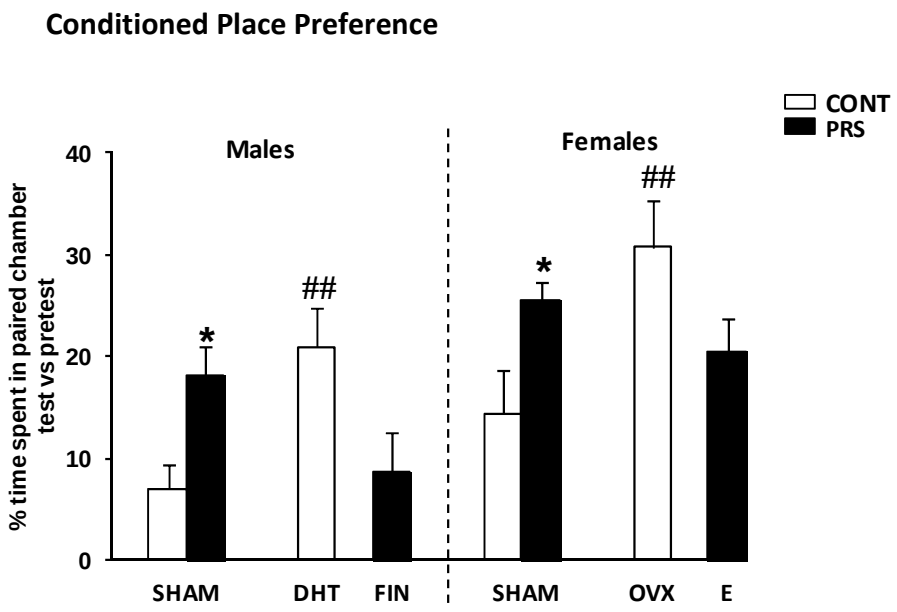

Fig. 4- Impact of sex hormones on cocaine-induced Conditioned Place Preference.

One month after surgeries and hormonal supplementations, rats were tested for their preference for cocaine, represented by the percentage of time spent in the cocaine-paired chamber during the test minus the pretest (A). Values are means ± SEM of 7-8 rats per group, *p<0.05 vs control animals of the same sex, # p<0.05 vs sham-operated animals (SHAM) of the same group (Control or PRS) and sex. ## p<0.01 vs sham-operated animals (SHAM) of the same group (Control or PRS) and sex. CONT=Control unstressed rats. DHT=dihydrotestosterone-supplemented, OVX=ovariectomized, E=estradiol-supplemented, FIN=finasteride-supplemented.

4- Sex hormones mediate the effect of early life stress on natural reward

In this part, we wanted to extend our study on cocaine to a particular aspect of addiction, namely addiction to natural reward chocolate. A better understanding of a high sensitiveness to foods or behaviors appeared indeed important for the complete understanding of disruptions of the reward pathway (Kelley and Berridge, 2002) in the PRS model.

Moreover, in a context where addiction to high palatable food like chocolate still receives poor recognition, and where there are only few studies using conditioned place preference, it was important in a well-known model of sensitiveness to drugs of abuse, to bring further elements in favor of this hypothesis, and to make a comparison between a strong psychostimulant, such as cocaine and chocolate, in the same protocol of measurement of drug-induced conditioned place preference.

Submitted

SEX HORMONES MEDIATE THE EFFECT OF EARLY LIFE STRESS ON NATURAL REWARD

Marie-Line Reynaert[1,8], Jordan Marrocco[2], Luana Lionetto[3], Lucie Deruyter[1,8], Delphine Allorge[4], Anna Moles[5,6], Stefania Maccari[1,8#], Sara Morley-Fletcher[1,8], Gilles Van Camp[1,8]*, Ferdinando Nicoletti[7,8]*

[1]UMR 8576/UGSF-Neuroplasticity Team, CNRS/University Lille1, Lille, France;
[2]IRCCS, Centro Neurolesi "Bonino-Pulejo", Messina, Italy;
[3]Advanced Molecular Diagnostic, Sant'Andrea hospital, Rome, Italy;
[4]EA4483, University Lille2, Lille, France;
[5]Institute of Neuroscience, National Research Council (C.N.R.), Rome, Italy;
[6]Genomnia, Lainate, Milano, Italy;
[7]IRCCS, Neuromed, Pozzili and Dept. of Pharmacology, Sapienza University of Rome, Italy
[8]LIA, International Associated Laboratory "Prenatal stress and neurodegenerative disorders" University of Lille 1/CNRS, Villeneuve d'Ascq, France; Neuromed, Pozilli, Italy; Sapienza University of Rome, Italy.

***These authors contributed equally to the work**

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[#]Corresponding Author
Prof Stefania Maccari
University Lille 1, France
Neuroplasticity Team - CNRS UMR 8576/ UGSF
Structural and Functional Glycobiology Unit, Co-director LIA
Bât C9, Avenue Mendeleïev -59655 Villeneuve d'Ascq France
Office: +33.32033.6042; Fax +33.32043.6555
stefania.maccari@univ-lille1.fr

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Abstract

We used adult rats subjected to prenatal restraint stress (PRS) to study the influence of early life stress and gender on hedonic sensitivity to natural rewards. In unstressed control rats we found a strong sex dimorphism in conditioned place preference for palatable food, with females showing a higher preference. Remarkably, PRS abolished sex dimorphism by enhancing food preference in males and reducing preference in females. PRS had profound effects on plasma levels of gonadal steroids in both sexes, enhancing dihydrotestosterone (DHT) levels in males and reducing 17β-estradiol (E_2) levels in females. Changes in DHT and E_2 levels were causally related to changes in food preference. In males, DHT supplementation enhanced food preference in unstressed rats, whereas inhibition of DHT synthesis reduced food preference in PRS rats. In females, ovariectomy reduced food preference in unstressed rats, and E_2 supplementation enhanced food preference in PRS rats. As biochemical surrogates, we measured the transcripts of selected genes in the hypothalamus, and monoamine levels in the nucleus accumbens (NAc) and prefrontal cortex (PFC). Changes in estrogen receptor mRNA levels in the hypothalamus and dopamine levels in the NAc were related to DHT and food preference in males. In contrast, changes in 5-HT_{2C} receptor mRNA levels in the hypothalamus and serotonin levels in the PFC were related to E_2 and food preference in females. These findings indicate that early life stress and gender are interdependent variables, and that changes in gonadal steroids sustain the effect of early life stress on hedonic preference for natural rewards.

Introduction

Drugs of abuse target the mesolimbic dopaminergic system and other brain reward circuits that have evolved to respond to natural rewards, such as food and sex. Of note, palatable food and drugs of abuse stimulate the reward system in similar ways (Rada et al., 2005; Olausson et al., 2006). Cocaine-dependent rats prefer sweetened water over intravenous cocaine (Lenoir et al., 2007; Avena et al., 2008). Therefore, understanding the mechanisms that regulate responses to natural rewards may provide new insights into the pathophysiology and treatment of drug abuse (Kelley and Berridge, 2002).

A number of variables influence hedonic sensitivity to natural rewards, such as context, gender, and early life events. Environmental cues paired with rewards acquire incentive salience *via* Pavlovian learning, and stimulate reward-seeking behavior. "Cue reactive" individuals are more vulnerable to develop impulse control disorders, such as addiction and binge eating (Saunders and Robinson 2013; Holden, 2001). Here, we used conditioned place preference as a method to measure the motivational effects of palatable food (Ventura et al., 2012).

There are only a few studies on the effect of gender on food rewarding. Endogenous β-endorphin is essential for motivation to food reward only in male mice (Hayward and Law, 2007), whereas prior access to a sweet is more protective against cocaine self-administration in female than male rats (Cason and Grigson, 2013). In addition, the effect of palatable food on gene expression in the mesolimbic system (Ong et al., 2013) and the phenomenon of palatable meal anticipation in mice (Hsu et al., 2010) are both gender-dependent.

Early life events have a profound impact on the developmental programming of the reward system. In rats, neonatal maternal separation enhances acquisition and maintenance of cocaine self-administration and food responding (Matthews et al., 1999; Kosten et al., 2004; Zhang et al., 2005), and an unstable maternal environment impairs the natural propensity to seek pleasurable sources of reward (Ventura et al., 2012).

How gender and early life events interact in shaping the sensitivity to natural rewards is unknown. We attempted to address this question by using the prenatal restraint stress (PRS) model in rats. Adult “PRS rats”, i.e. the offspring of dams exposed to repeated episodes of restraint stress during pregnancy, show biochemical and behavioral abnormalities that are indicative of an anxious/depressive phenotype (Morley-Fletcher et al., 2011; Laloux et al., 2012; Mairesse et al., 2013; Marrocco et al., 2012; 2014), and also show an enhanced vulnerability toward addiction (Deminière et al., 1992; Koehl et al., 2000; Morley-Fletcher et al., 2004; Van Waes et al., 2009; Kippin et al., 2008). Some of the hallmarks of PRS rats, such as anxiety-like behavior and

changes in hippocampal neuroplasticity, are gender-dependent (Zuena et al., 2008), whereas others, such as depression-like behavior, are not (Koehl et al., 1999; Van Waes et al., 2011).

We now report that prenatal stress and gender are two tightly dependent variables in shaping hedonic sensitivity in the adult life, and that the effect of PRS on place preference for palatable food is sustained by profound changes in the levels of sex steroids in both males and females.

Materials and Methods

Animals

Adult female Sprague-Dawley rats weighing about 250 g and sexually-experienced males (400-500 g) were purchased from Charles River Laboratories (L'Arbresle Cedex FRANCE). Females were group-housed for 3 weeks for acclimation and oestrous cycle coordination in a temperature (22+/-2°C) and humidity-controlled room under a 12h light-dark cycle with lights off at 20h. Males were single-housed during this period. Water and chow were provided *ad libitum*. Then, females were placed with a male for a night and the day corresponding to spermatozoids revealing by microscopy or copulation plug visualisation was designated as embryonic day 0 (E0), and females were housed individually in transparent Plexiglas cages and randomly assigned to control or stressed group (n=14 per group).

Prenatal restraint stress procedure

Pregnant females were subjected to restraint stress according to our standard protocol (Maccari et al., 1995; Morley-Fletcher et al., 2003). At E11 of pregnancy until delivery, female rats were submitted to three stress sessions daily (45 min each), during which they were placed in transparent plastic cylinders and exposed to bright light or were left undisturbed (control dams).

Only rats from litters of 10-14 rats with a similar number of males and females were used. After weaning (P21), offspring grew up (2-3 brothers/2-3 sisters per cage) until reaching the good stage (adulthood) to be used for experimentation. A maximum of two animals was used for a same group to avoid any litter effect (Becker and Kowall, 1977; Chapman and Stern, 1979). All experiments followed the rules of the European Communities Council Directive 86/609/EEC.

The local ethics committee approved the prenatal stress procedure. Separate sets of adult rats (3-4 months of age) were used for surgeries and/or behavioral and hormonal analyses. The time line of experiments is depicted in figure 1.

Surgical Procedures

To assess the effect of sexual hormones on rats preference for high palatable food as natural reinforcement, many hormonal modulations were carried out. Male and female rats (6-9 per group) were anesthetized with 1 mL/kg of a solution of ketamine hydrochloride (100 mg/kg IP), xylazine (8 mg/kg IP) and acepromazine (1 mg/kg IP), and one of the following procedures was performed (ovariectomy (OVX), orchidectomy (ORX), sham ovariectomy/orchidectomy (SO). Ovariectomies consisted in bilateral dorsal incisions (1.0-1.5 cm), 2 cm below the last rib, and for orchidectomy, testis and epididymis were removed *via* a small incision at the tip of the scrotum.

After removal of the gonads, the remaining tissue was replaced into the peritoneal cavity and the skin incision closed with suture. Betadine was applied to ensure the appropriate asepsis and antisepsis. Sham surgeries were carried out anesthetizing the animals and making incisions, but not removing gonads.

For the hormonal study, 20 mm and 10 mm (for replacement and supplementation treatment respectively) silastic tube implants (inner diameter 1,98mm; outer diameter 3.18 mm; Biesterfeld France, Rueil-Malmaison Cedex, France) were placed subcutaneously in the midscapular region during surgery. Empty implants were used as control in gonadectomized and sham-operated rats, whereas rats with hormonal replacement or supplementation were sham-operated and implanted with capsules filled either with testosterone (T), dihydrotestosterone (DHT), estradiol benzoate (E_2) or finasteride (FIN). All products were provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Implants were expected to supply the hormones for two months at a level close to the physiological one (Van Coppenolle et al., 2001).

Hormone measurement

Trunk blood was collected after the end of experiment 1 to measure hormones at basal level and at the end of experiment 2 to assess the effect of gonadectomy and hormones replacement/supplementation on levels of gonadal hormones. Animals were killed one week after CPP procedure.

Blood was collected in tubes containing EDTA 6%, and samples (n=5-8 rats per group) were centrifuged for 15 min, at 4°C, 1500 g. to isolate plasma. Hormones levels were determined using ELISA kits (Demeditec Diagnostics, Kiel, Germany). Samples were analyzed in duplicate. The sensitivity of tests was 0,066 ng/ml, 9,714 pg/mL and 6 pg/mL, respectively for T, E_2 and DHT.

Conditioned Place Preference (CPP)

Conditioned place preference was used to study sensitiveness to food reward, as described by Ventura et al. (2012) with minor modifications. Standard diet was used as neutral stimulus, whereas high palatable food milk chocolate was used as rewarding stimulus (for both foods, 5 grams were delivered during each conditioning session). Experiment 1 evaluated the influence of PRS and sex differences in adult (4 mo old) male and female control and PRS rats (n=7-8 rats per group). Experiment 2 assessed the influence of sex hormones manipulation and PRS in adult control and PRS male and female rats operated at 3 months of age, then tested at 4 months of age after one month of recovery and hormonal assessment (n=6-9 rats per group).

CPP procedure always began after two hours of food deprivation. The CPP apparatus was made in opaque Plexiglas and had two compartments with different associated visual cues (one chamber white and the other grey). On day 1 (habituation), animals were exposed to milk chocolate for 2 hours. On day 2 (pretest), rats were allowed to explore the whole apparatus during 20 minutes in the absence of food stimulus, in order to determine their spontaneous preference for one chamber of the apparatus. Conditioning (8 days, 30 min/session) with reward was conducted in the least preferred side. During conditioning, animals were alternatively exposed to milk chocolate or standard diet as neutral stimulus. Day 11 (test) was performed as pretest. Data were expressed as a difference of percentage of time spent in rewarding stimulus-paired chamber during the test minus the pretest.

Gene expression analysis by Taqman

In order to investigate a correlation between behavioral data in CPP for chocolate and stress-/sex-induced modifications of key genes involved in stress regulation, motivational system, hedonic feeding, and hormonal regulation, Taqman Real Time PCR was carried out in the hypothalamus of rats belonging to the various experimental groups. One week after CPP test, rats were sacrificed and brain areas of interest were kept frozen under -80°C until use.

RNA extraction was performed on hypothalamus samples (n=4-5) using RNeasy Plus mini kit (Qiagen, Courtaboeuf, France). Concentration of RNA samples was assed using Nanodrop (ND-1000, Labtech, Nettetal, Allemagne), and quality verified by Rin (RNA Integrity Number; Bioanalyzer 2100, Agilent Technologies, Les Ulis, France).

Appropriated dilutions were performed in order to have 1 µg RNA/10 µL sample. Retrotranscription was carried out with High-Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, France).

Transcript levels were then measured by real-time PCR using TaqMan assays (Applied Biosystems) and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Acquisition of data was performed by means of a StepOnePlusTM software, and data were obtained as Ct (threshold cycle). Then, a ΔCt (Ct of the considered gene - Ct of the GAPDH gene) and a ΔΔCt (Ct of a particular gene - Ct of the same gene in unstressed sham-operated rats) were calculated. Finally, the calculus $2^{-\Delta\Delta Ct}$ allowed us to express data as genes expression RQ (for relative quantitation) in each group in comparison to Control SO males.

Measurement of steady-state levels of monoamines in the nucleus accumbens (NAc) and prefrontal cortex (PFC).

Catecholamines and serotonin analysis were performed on NAc and PFC samples of rats belonging to the various experimental groups using the Catecholamine Dual Kit and Serotonin kit (Eureka srl, Chiaravalle, Italy) according to the manufacturer's instructions. Briefly, tissue samples were weighted and homogenized in 500 µL of TCA 4%, distributed in aliquots and stored at -80°C until analysis. Both chromatographic separations were carried out on a reversed-phase column (150 x 2.0 mm, Luna C18, 5 µm, 100 Å pore size, Phenomenex, Torrance, CA, USA) equipped with a security guard precolumn (Phenomenex) containing the same packing material. The Liquid Chromatography System was composed by a binary pump (LabFlow 4000, LabService Analytica, Anzola Emilia, Italy), a dynamic mixer (811C, Gilson, Middleton, WI, USA), an autosampler (Model 231, Gilson), and a column oven (LabService Analytica). The column was maintained at room temperature. The injection volume were 100 µl and the total analysis run time were 20 min. Analytes were detected by a fluorescence detector (ProStar, Varian, Walnut Creek, CA, USA; excitation wavelength=360 nm and emission wavelength=490 nm for catecholamines; excitation wavelength=285 nm and emission wavelength=344 nm for serotonin). Data analysis was performed using the Star Workstation software version 6.20 (Varian). Data were expressed as monoamines steady-state levels (pg) per milligram of weight sample in table 2, and represented as RQ (relative quantitation) in comparison to the respective unstressed sham-operated rats in Fig. 6.

Statistical analysis

Statistical analysis was performed by Student's t test (Fig. 2B,C), one-way ANOVA followed by Fisher's PLSD (Fig. 3-6), or two-way ANOVA followed by Fisher's PLSD (Fig. 2A; Tables 1 and 2). A p value < 0.05 was considered as significant.

Results

PRS abolished the gender effect on hedonic preference for a natural reward

We examined the behavior of adult PRS and unstressed male and female rats in the conditioned place preference for palatable food. We found a clear-cut sex dimorphism in unstressed control rats with higher preference for palatable food in females than in males. PRS enhanced preference in males and reduced preference in females (ANOVA, group x sex, $F_{(1,26)}$=12.26, p<0.01), thereby eliminating sex dimorphism (**Fig. 2A**).

We measured plasma levels of gonadal steroid hormones in unstressed and PRS rats of both sexes. We found that PRS halved T levels and almost doubled DHT levels in male rats (T, t=5.26, df=12, p<0.01; DHT, t=2.86, df=13, p<0.05) (**Fig. 2B)**. E_2 levels were very low in males and did not differ between unstressed and PRS rats (7.0 $\pm$ 2.05 and 8.3 $\pm$ 2.4 pg/ml, respectively; n = 6). In females, PRS caused a 70% reduction in E_2 levels (t=2.22, df=13, p<0.05) (**Fig. 2C**). Thus, PRS reduced T to DHT ratio in males and caused hypoestrogenism in females.

Correction of hormonal imbalance abrogated the effect of PRS on hedonic sensitivity.

a) Male rats.

We specifically examined whether either the increase in DHT or the associated reduction in T levels could account for the greater hedonic preference of male PRS rats. We used the following experimental groups: (i) orchidectomized PRS rats implanted with empty silastic tubes; (ii) sham-operated (SO) PRS rats implanted with either empty tubes, tubes filled with T, or tubes filled with the 5α-reductase inhibitor, finasteride; (iii) orchidectomized unstressed rats implanted with empty tubes or with tubes filled with T; and (iv) SO unstressed rats implanted with empty tubes or with tubes filled with DHT.

Hedonic preference is shown in **Fig. 3A** (ANOVA, $F_{(6,50)}$=5.35, p<0.01); androgen levels are shown in **Fig. 3B** (T, ANOVA, $F_{(3,26)}$=14,34, p<0.01; DHT, $F_{(4,32)}$=16,14, p<0.01). Data in SO rats implanted with empty tubes were identical to those found in intact rats, with PRS rats showing greater hedonic sensitivity (p<0.05), lower T levels (p<0.01), and greater DHT levels (p<0.05), as compared to unstressed controls. Orchidectomy in PRS rats caused the expected drop in DHT levels (p<0.01) and abolished hedonic preference (p<0.05). The same was found in SO PRS rats treated with finasteride (DHT levels, p<0.01; hedonic preference, p<0.01), suggesting that DHT was responsible for the greater preference for palatable food in PRS rats.

In contrast, the reduction of T had no role in hedonic sensitivity because T supplementation in PRS rats caused no significant changes in place preference.

Hormonal manipulation in unstressed rats fully confirmed the critical role of DHT in hedonic preference. Accordingly, SO unstressed rats supplemented with DHT showed increased plasma levels of DHT ($p<0.01$) and displayed the same hedonic preference as PRS rats (SO unstressed vs. SO + DHT, $p<0.01$). Orchidectomy in control rats had no effect on food preference; T replacement in orchidectomized control had also no effect on behavior although it raised plasma T levels to the same levels found in SO PRS rats.

b) Female rats

We used the following experimental groups: (i) SO PRS rats implanted with empty tubes or with tubes filled with E_2; (ii) ovariectomized unstressed rats implanted with empty tubes or with tubes filled with E_2; and (iii) SO unstressed rats implanted with empty tubes. Hedonic preference is shown in **Fig. 4A** (ANOVA, $F_{(3,33)}=5.22$, $p<0.01$); E_2 levels are shown in **Fig. 4B** (ANOVA, $F_{(3,28)}=37.65$, $p<0.01$). Data in SO rats implanted with empty tubes were identical to those found in intact rats, with female PRS rats showing lower hedonic sensitivity ($p<0.05$) and lower E_2 levels ($p<0.05$), as compared to female unstressed rats. E_2 supplementation in SO PRS rats markedly enhanced plasma E_2 levels ($p<0.01$) and enhanced hedonic sensitivity to the same levels observed in unstressed rats (PRS SO vs. PRS+ E_2, $p<0.05$). Ovariectomy in unstressed rats caused the expected drop in E_2 levels and nearly abolished hedonic preference ($p<0.01$). E_2 replacement in ovariectomized unstressed rats markedly enhanced E_2 levels ($p<0.01$) and restored hedonic preference. These data indicate that E_2 has a strong impact on hedonic preference in female rats, and that PRS female rats show a reduced hedonic preference because of the lower estrogen levels.

Gender and PRS effect on gene expression profile in the hypothalamus

We performed TaqMan analysis of 25 genes related to sex differentiation, incentive motivation, hedonic feeding, and regulation of the hypothalamic pituitary adrenal (HPA) axis in the hypothalamus of unstressed and PRS rats of both sexes. Analysis was carried out on 4-5 SO rats implanted with empty tubes used in experiment 2. Only four genes (encoding for ERα, ERβ, serotonin 5-HT_{2C} receptor, and cocaine-and-amphetamine receptor transcript peptide or CARTP) showed changes related to sex, PRS, or both (**Table 1**). PRS increased ERα, ERβ, and CARTP mRNA levels in males, and 5-HT_{2C} receptor mRNA levels in females. Sex dimorphism was also found, with unstressed females showing lower ERβ and 5-

HT_{2C} receptor mRNA levels as compared to unstressed males. In contrast, PRS females showed lower ERα, ERβ, and CARTP, and higher 5-HT_{2C} receptor mRNA levels as compared to PRS males **(Table 1)**. Two-way ANOVA: ERα mRNA levels (group x sex, $F(1,16)=3.90$, $p=0.06$); ERβ mRNA levels (group effect, $F(1,16)=13.22$, $p<0.01$; sex effect, $F(1,16)=123.02$, $p<0.01$); 5-HT2C receptor mRNA levels (group x sex, $F(1,16)=18.36$, $p<0.01$); and CARTP mRNA levels (group x sex, $F(1,16)=7.58$, $p<0.05$).

We extended the analysis of these four genes to selected groups of 4-5 animals subjected to hormonal manipulation and used for behavioral analysis, i.e. unstressed males treated with DHT, PRS males treated with finasteride, unstressed females subjected to ovariectomy, and PRS females rats treated with E2.

a) Effect of hormonal manipulation in males

Treatment with DHT significantly increased ERβ and reduced 5-HT2C receptor mRNA levels in unstressed rats. Levels of both transcripts in unstressed rats treated with DHT were similar to those found in untreated PRS rats. In contrast, treatment of PRS rats with finasteride substantially reduced ERα, ERβ, and CARTP mRNA levels. There was no significant difference between ERα, ERβ, and CARTP mRNA levels between PRS rats treated with finasteride and untreated unstressed rats (ANOVA, ERα mRNA levels, $F(2,16)=9.79$, $p<0.01$; ERβ mRNA levels, $F(2,16)=19.14$, $p<0.01$; 5-HT2C receptor mRNA levels, $F(2,16)=8.90$, $p<0.01$; CARTP mRNA levels, $F(2,16)=18.71$, $p<0.01$) **(Fig. 5A)**.

b) Effect of hormonal manipulation in females

Ovariectomy significantly enhanced 5-HT2C receptor, CARTP, and leptin receptor mRNA levels in unstressed rats, whereas estrogen treatment substantially reduced 5-HT2C receptor and CARTP mRNA levels in PRS rats (ANOVA, 5-HT2C receptor mRNA levels, $F(2,16)=6.56$, $p<0.01$; CARTP mRNA levels, $F(2,16)=9.57$, $p<0.01$; leptin receptor mRNA levels, $F(2,16)=5.84$, $p<0.05$ **(Fig. 5B)**.

Gender and PRS effect on steady-state monoamine levels in the nucleus accumbens (NAc) and prefrontal cortex (PFC).

Steady-state levels of dopamine (DA), 5-HT, noradrenaline (NA), and adrenaline (A) were measured in 4-5 animals of the same groups used for mRNA and behavioral analysis (see above). Like in behavioral analysis, monoamine levels showed a remarkable sex dimorphism

in the NAc, with much greater values being observed in females. In the PFC, female PRS rats showed higher NA and DA levels as compared to male PRS rats, whereas unstressed females showed higher 5-HT levels as compared to unstressed males. In females, PRS caused a significant reduction of all monoamines in the NAc, a reduction in 5-HT levels in the PFC, and an increase in NA and DA levels in the PFC (Table 2). Two-way ANOVA: NAc NA levels (ANOVA, group x sex, $F(1,15)=6.83$, $p<0.05$); NAc A levels (group x sex, $F(1,15)=9.84$, $p<0.01$); NAc DA levels (sex effect, $F(1,15)=9.21$, $p<0.01$); NAc 5-HT levels (group effect, $F(1,15)=7.17$, $p<0.05$; sex effect, $F(1,15)=10.99$, $p<0.01$); PFC NA levels (sex effect, $F(1,14)=11.23$, $p<0.01$), PFC DA levels (group x sex, $F(1,15)=5.64$, $p<0.05$), PFC 5-HT levels (group effect, $F(1,14)=9.63$, $p<0.01$; sex effect, $F(1,14)=10.08$, $p<0.01$, group x sex, $F(1,14)=3.88$, $p=0.06$).

In males, PRS did not induce significant changes in monoamine levels at least when statistical analysis included all values obtained in female rats **(Table 2)**.

a) Effect of hormonal manipulation on monoamine levels in males

The following groups were compared: SO unstressed and PRS rats implanted with empty tubes; SO unstressed rats treated with DHT; and SO PRS rats treated with finasteride. Here, two-way ANOVA revealed a significant impact of PRS on DA and 5-HT levels in the NAc, and 5-HT levels in the PFC **(Fig. 6A)**.

PRS enhanced DA levels in the NAc, an effect fully reversed by treatment with finasteride (ANOVA, $F(2,15)=7.34$; $p<0.01$). As opposed to DA levels, 5-HT levels were substantially reduced by PRS in the NAc, and this effect was partially reversed by finasteride. DHT supplementation in unstressed rats reduced 5-HT to the same levels found in untreated PRS rats (ANOVA, $F(2,16)=10.93$, $p<0.01$). Thus changes in NAc DA levels fitted nicely with changes in hedonic sensitivity and serum DHT levels, whereas changes in NAc 5-HT levels were inversely related.

NA and A levels in the NAc showed significant changes only in PRS rats treated with finasteride (ANOVA, $F(2,16)=12.27$, $p<0.01$; and $F(2,16)=7.96$, $p<0.01$, respectively) **(Fig. 6A)**.

In the PFC, changes in DA levels were not statistically significant. In contrast, PRS caused a significant reduction in 5-HT levels, and this effect was reversed by finasteride. DHT treatment in unstressed rats also reduced PFC 5-HT levels to a greater extent as compared to untreated PRS rats (ANOVA, $F(2,16)=11.93$, $p<0.01$) **(Fig. 6A)**. 5-HT levels were inversely related to hedonic sensitivity and DHT also in the PFC.

b) Effect of hormonal manipulation on monoamine levels in females

In the NAc, NA, DA, and 5-HT levels were significantly reduced in untreated PRS rats and in ovariectomized unstressed rats (ANOVA, NA, $F(2,15)=15.27$, $p<0.01$; DA, $F(2,15)=11.72$, $p<0.01$, 5HT, $F(2,15)=9.82$, $p<0.01$). However, as opposed to behavioral data, estrogen supplementation was unable to reverse changes in NA, DA, and 5-HT levels induced by PRS. A levels were significantly reduced in ovariectomized unstressed rats and, in PRS rats, supplementation with E2 corrected the deficit (A, $F(2,15)=36.74$, $p<0.01$) (Fig. 6B). In the PFC of unstressed rats, ovariectomy caused large increases in NA and DA levels, with values being even higher than those found in untreated PRS rats. In contrast, both ovariectomy and PRS substantially decreased 5-HT levels. E_2 treatment reversed the effect of PRS on 5-HT levels in the PFC (ANOVA, NA, $F_{(2,13)}=6.57$, $p<0.01$; DA, $F_{(2,13)}=5.74$, $p<0.05$, 5HT, $F_{(2,13)}=8.94$, $p<0.01$) (**Fig. 6B**).

Discussion

The main finding of this study is that gender and early life stress are two interdependent variables in shaping hedonic sensitivity to natural rewards in the adult life. Our data indicate that gonadal steroids are essential for the maintenance of the hedonic phenotype in the adult life, and that PRS alters hedonic sensitivity by causing permanent changes in the secretion of sex hormones. To our knowledge, this is the first time that hedonic sensitivity to natural rewards has been examined in the PRS rat model. PRS rats are more prone to become addicted to psychostimulants (Deminière et al., 1992; Koehl et al., 2000; Morley-Fletcher et al., 2004; Kippin et al., 2008), but no in-depth analysis of the gender effect on drug-seeking behavior has been performed in these animals. Because palatable food and drugs of abuse stimulate the reward system in the same ways (Rada et al., 2005; Olausson et al., 2006), our findings may lay the groundwork for the study of the interaction between early life stress and gender/sex steroids in models of addiction.

The female preference for palatable food we have seen in unstressed rats was not unexpected (Roth et al., 2004; Klump et al., 2013). PRS abolished sex dimorphism on hedonic sensitivity by enhancing place preference in males and reducing place preference in females. We wish to highlight that there were no changes in food consumption, indicating that early life stress and gender had a specific impact on food reward.

In males, PRS substantially reduced plasma T levels (see also Weisz et al., 1982) and enhanced DHT levels. This is in line with the greater activity of 5α-reductase (the enzyme that converts T into DHT) found in PRS rats (Reznikov et al., 2001; Ordyan and Pivina, 2005; Reznikov and Tarasenko, 2007). In females, PRS caused a marked reduction in serum E_2 levels, in agreement with a recent report (Ordyan et al., 2013).

So far, studies of the interaction between early life stress and sex hormones have focused on the perinatal period (Pereira et al., 2006; Morgan and Bale, 2011). Here, instead, we adopted a series of strategies aimed at rebalancing sex hormones in adult PRS rats, and, therefore, we did not interfere with the developmental programming driven by PRS and sex steroids. We found that DHT supplementation in unstressed male rats enhanced hedonic sensitivity to levels approximating those found in PRS rats, whereas inhibition of DHT synthesis with finasteride in male PRS rats completely reversed the increase in hedonic sensitivity. In contrast, hedonic sensitivity in PRS rats was not corrected by T supplementation. This demonstrated that the abnormal hedonic sensitivity to natural rewards in male PRS rats was caused by the increase in DHT levels, and not by the reduction in T levels. An interesting hypothesis for translational studies is that DHT formation supports drug-seeking behavior in athletes taking testosterone

esters, or that the use of 5α-reductase inhibitors in humans (e.g., for the treatment of benign prostatic hyperplasia or androgenetic alopecia) causes changes in hedonic sensitivity that are shaped by early life experiences.

In females, estrogens have an established role in regulating the activity of the brain reward system (Galankin et al., 2010), and E_2 replacement or treatment with estrogen receptor ligands fully reverse changes in drug-seeking behavior and responses to psychostimulants caused by ovariectomy (Lynch 2001; Lynch et al., 2006; Hu et al., 2004; Segarra et al., 2010; Jackson et al., 2006). Hence, only E_2 levels were measured in females in our study. We found that E_2 replacement reversed the lowering effect of ovariectomy on hedonic sensitivity in unstressed female rats. In addition, E_2 supplementation in PRS female rats enhanced hedonic sensitivity to the same levels found in unstressed rats. Again, this raises a number of interesting questions in humans, e.g. how early life stress affects ovarian estrogen production and associated hedonic sensitivity, or how estrogen treatment during fertility or after menopause affects responses to natural rewards.

We searched for potential biochemical correlates that could support behavioral data by measuring the transcripts of a battery of selected genes in the hypothalamus, and monoamine levels in the NAc and PFC. In the hypothalamus of male rats, changes in the transcripts of ERα and ERβ paralleled changes in hedonic sensitivity and DHT levels under basal conditions and following hormonal manipulations. The increase in both transcripts found in the hypothalamus of PRS rats is not in agreement with a previous report showing that PRS enhances hypothalamic ER mRNA levels in the early neonatal period, but not in the adulthood (Henry et al., 1996). Expression of ERα and -β mRNA was under the control of DHT because it was affected by treatment with finasteride in PRS rats or by DHT supplementation in unstressed rats. DHT is known to activate ERβ in the hypothalamus *via* its metabolite, 5α-androstane 3β,17β diol (Lund et al., 2006; Handa et al., 2008). In addition, both DHT and 5α-androstane 3β,17β diol enhance ERβ expression in prostatic tissue of castrated rats (Oliveira et al., 2007). Thus, DHT or its diol metabolite might interact with ER β in the hypothalamus, thereby regulating the expression of ERs.

In females, we were surprised to find no changes in ER expression in relation to hormonal modification and hedonic behavior. In contrast, changes between hypothalamic 5-HT2C receptor mRNA levels and hedonic sensitivity were inversely related. Accordingly, 5-HT2C receptor mRNA levels were increased in female PRS rats, which displayed a lower preference for palatable food. Ovariectomy in unstressed rats enhanced the transcript of 5-HT2C receptors and reduced hedonic sensitivity, whereas E_2 supplementation in PRS rats lowered the transcript of 5-HT2C receptors and increased hedonic sensitivity. These findings are in nice agreement with the

aversive and anti-reward properties of 5-HT2C receptor agonists (Grottick et al., 2001; Mosher et al., 2006) and with the increased drug-seeking behavior displayed by 5-HT2C knockout mice (Rocha et al., 2002; Chou-Green et al., 2003).

We also measured endogenous monoamine levels without the aid of radioactive tracing or metabolic inhibitors, and, therefore, measurements were not informative about the activity of monoaminergic pathways. In spite of these limitations, we found gender-dependent changes caused by PRS and/or hormonal manipulations that were related to place preference for palatable food. Measurements were performed in the NAc and PFC because monoaminergic transmission in these two regions is tightly linked to food reward (Kelley and Berridge, 2002; Fallon et al., 2007). In the NAc of male rats, PRS caused an increase in DA levels, which was reversed by finasteride and mimicked by DHT supplementation in unstressed rats. In contrast, changes in 5-HT levels in the NAc and PFC were inversely related to DHT levels and hedonic sensitivity. This suggests that early life stress in males alters the DA/5-HT balance in the NAc via a sustained increase in DHT production. DHT might have a direct action on monoaminergic transmission in the NAc because direct implant of the hormone in the NAc shell exerts rewarding properties (Frye et al., 2002). Alternatively, DHT might influence the activity of hypothalamic neuropeptides, such as ghrelin and orexin, thereby causing secondary changes in monoaminergic transmission in the NAc that are related to the reinforcing properties of food (Patyal et al., 2012; Kawahara et al., 2013).

In females, a better correlation between changes in monoamines and hedonic behavior was found in the PFC, where ovariectomy and PRS caused similar changes in 5-HT levels, and the PRS phenotype was reversed by estrogen supplementation. Activation of PFC serotonergic transmission by estrogen has been associated to memory and cognition enhancement both in rodents and humans (Maki, 2005; Maki and Dumas, 2009; Inagaki et al., 2010; Jacome et al., 2010; Epperson et al., 2012). Interestingly, estrogen and DHT have been found to exert an opposite modulation on 5-HT turnover rate in the medial PFC (Handa et al., 1997), which fits nicely with our data obtained in male and female rats. The precise role of PFC serotonin transmission in food rewarding remains to be investigated.

In conclusion, we have shown that gender-dependent changes in gonadal steroids sustain the effect of early life stress on hedonic preference for natural rewards. Physiological or pharmacological modifications of the hormonal status in the adulthood might profoundly affect hedonic sensitivity in a manner that is tightly dependent on early life experiences. This may lay the groundwork for novel therapeutic strategies aimed at restoring the appropriate response to natural rewards in stress-related disorders, such as depression and drug addiction.

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Legends

Fig. 1 - Experimental design.
Experiment 1 was planned to examine the effect of PRS and gender on sensitivity to high palatable food (milk chocolate) in a conditioned place preference paradigm in adult rats. *Experiment 2* was planned to study the effect of gonadectomy and hormonal supplementation/replacement with implants filled with testosterone (T), dihydrotestosterone (DHT), or finasteride (FIN) in males, and with estradiol (E_2) in females, on food preference. Surgeries were performed at 3 months of age and animals were tested one month later. Trunk blood was collected at the end of experiments 1 and 2 for hormonal measurements. Animals were killed one week after behavioral procedure. CONT = unstressed control rats; SO = sham-operated rats; ORX = orchidectomized rats; OVX = ovariectomized rats.

Fig. 2 – Effect of early life stress and gender on food preference and sex steroids in adult rats.
Conditioned place preference for palatable food in unstressed (CONT) or PRS rats of both sexes is shown in (A). Plasma levels of sex hormones are shown in (B). Levels of testosterone (T), dihydrotestosterone (DHT) and estradiol (E_2) were measured one week after the end of the conditioning procedure. Values are means ± S.E.M of 7-8 determinations. $p<0.05$ vs. the respective CONT group (*) or vs. the respective groups (PRS or CONT) of different sex (#) (Two-way ANOVA + Fisher's PLSD in A, and Student's t test in B).

Fig. 3 – Effect of hormonal manipulations on food preference and gonadal steroids in male unstressed or PRS rats.
Food preference and plasma levels of gonadal steroids are shown in (A) and (B), respectively. Values are means ± S.E.M of 5-8 determinations. $p<0.05$ vs. the respective CONT group (*) or vs. sham-operated (SO) animals of the same group (PRS or CONT) (#) (One-way ANOVA + Fisher's PLSD). CONT = unstressed control rats; T = testosterone-supplemented/replaced rats; DHT = dihydrotestosterone-supplemented rats, FIN = finasteride-treated rats, ORX = orchidectomized rats.

Fig. 4 – Effect of hormonal manipulations on food preference and gonadal steroids in female unstressed or PRS rats.

Food preference and plasma levels of gonadal steroids are shown in (A) and (B), respectively. Values are means ± S.E.M of 5-9 determinations. $p<0.05$ vs. the respective CONT group (*) or vs. sham-operated (SO) animals of the same group (PRS or CONT) (#) (One-way ANOVA + Fisher's PLSD). OVX = ovariectomized rats; E_2 = estradiol supplemented/replaced rats

Fig. 5 – Effect of early life stress and gender on the expression of selected genes in the hypothalamus.

mRNA levels of the selected genes in males and females rats are shown in (A) and (B), respectively. Data are expressed as fold changes vs. sham-operated (SO) CONT rats and are means ± S.E.M of 4-5 determinations. $p<0.05$ vs. SO CONT unstressed rats (*), vs. SO PRS rats (#) or vs. SO CONT treated with DHT ($) (One-way ANOVA + Fisher's PLSD). CONT = unstressed control rats; DHT = dihydrotestosterone-supplemented rats; FIN = finasteride-treated rats; OVX = ovariectomized rats; E_2= estradiol-supplemented rats.

Fig. 6 – Effect of early life stress and gender on monoamine levels in the nucleus accumbens (NAc) and prefrontal cortex (PFC).

Dopamine (DA), noradrenaline (NA), adrenaline (A), and serotonin (5-HT) levels in males and females are shown in (A) and (B), respectively.

Data are expressed as fold changes vs. sham-operated (SO) CONT rats and are means $\pm$ S.E.M. of 4-5 determinations. $p<0.05$ vs. SO CONT rats (*), vs. SO PRS rats (#) or vs. SO CONT treated with DHT ($) (One-way ANOVA + Fisher's PLSD). CONT = unstressed control rats; DHT = dihydrotestosterone-supplemented rats; FIN = finasteride-treated rats; OVX = ovariectomized rats; E_2= estradiol-supplemented rats.

Table 1 - Effect of early life stress and gender on hypothalamic gene expression.

	Gene name	CONT ♂	PRS ♂	CONT ♀	PRS ♀	Taqman ID
Sex differentiation	ERα	1.00 ± 0.23	1.75 ± 0.15*#	1.18 ± 0.35	1.10 ± 0.10#	Rn01479215_g1
	ERβ	1.00 ± 0.13#	1.47 ± 0.10	0.69 ± 0.06	0.89 ± 0.09#	Rn02606541_m1
	AR	1.00 ± 0.16	0.98 ± 0.07	0.95 ± 0.15	0.93 ± 0.12	Rn00560747_m1
Motivational system	$5HT_{2C}R$	1.00 ± 0.05#	0.80 ± 0.03*#	0.73 ± 0.05#	1.12 ± 0.12 *#	Rn00562748_m1
	DA D1AR	1.00 ± 0.10	1.06 ± 0.12	1.07 ± 0.27	0.88 ± 0.09	Rn03062203_s1
	DA D2R	1.00 ± 0.08	1.01 ± 0.11	0.82 ± 0.13	0.78 ± 0.09	Rn00561126_m1
	Prodynorphin	1.00 ± 0.14	1.14 ± 0.23	0.64 ± 0.06	0.74 ± 0.11	Rn00571351_m1
	Protein kinase Cγ	1.00 ± 0.21	1.00 ± 0.19	0.77 ± 0.17	0.79 ± 0.07	Rn00574583_m1
	BDNF	1.00 ± 0.20	1.15 ± 0.13	1.05 ± 0.13	1.25 ± 0.15	Rn02531967_s1
	cFos	1.00 ± 0.15	1.34 ± 0.44	1.37 ± 0.56	1.37 ± 0.27	Rn00487426_g1
	Delta Fos B	1.00 ± 0.27	1.61 ± 0.38	2.83 ± 1.46	2.88 ± 1.44	Rn00500401_m1
	Tyrosine hydroxylase	1.00 ± 0.30	0.85 ± 0.07	1.05 ± 0.33	0.70 ± 0.08	Rn00562500_m1
Hedonic feeding	CARTP	1.00 ± 0.12	1.78 ± 0.16*#	0.99 ± 0.16	1.04 ± 0.08#	Rn01645174_m1
	Orexin	1.00 ± 0.16	1.44 ± 0.21	1.00 ± 0.25	1.66 ± 0.43	Rn00565995_m1
	Orexin R1	1.00 ± 0.12	1.18 ± 0.11	0.88 ± 0.24	0.84 ± 0.11	Rn00565032_m1
	Orexin R2	1.00 ± 0.06	1.03 ± 0.17	0.83 ± 0.09	1.01 ± 0.12	Rn00565155_m1
	Ghrelin	1.00 ± 0.16	1.13 ± 0.14	0.97 ± 0.15	1.05 ± 0.11	Rn01425835_m1
	Leptin R	1.00 ± 0.21	1.45 ± 0.32	0.77 ± 0.08	1.11 ± 0.09	Rn00561465_m1
	NPY	1.00 ± 0.16	1.13 ± 0.30	0.81 ± 0.15	1.22 ± 0.19	Rn01410146_m1
	Agouti related peptide	1.00 ± 0.21	0.96 ± 0.32	0.58 ± 0.14	0.74 ± 0.07	Rn01431703_g1
Stress regulation	CRH	1.00 ± 0.18	1.25 ± 0.15	0.84 ± 0.15	1.04 ± 0.15	Rn01462137_m1
	CRH R1	1.00 ± 0.16	1.06 ± 0.09	0.82 ± 0.16	0.78 ± 0.06	Rn00578611_m1
	CRH R2	1.00 ± 0.13	1.05 ± 0.13	0.70 ± 0.20	0.85 ± 0.06	Rn00575617_m1
	GR	1.00 ± 0.18	1.24 ± 0.12	0.87 ± 0.17	0.91 ± 0.10	Rn00561369_m1
	MR	1.00 ± 0.07	1.09 ± 0.07	0.83 ± 0.12	0.87 ± 0.06	Rn00565562_m1

Expression of selected genes in the hypothalamus of unstressed or PRS rats of both sexes was assayed by Custom Taqman qRT-PCR. The biological function of the genes is highlighted as indicated by Gene Ontology. Expression levels of each sample were normalized to the average levels of unstressed male rats and expressed as Relative Quantitation (RQ). Data are means ± S.E.M of 4-5 determinations. CONT = Control unstressed rats. $p<0.05$ (Two-way ANOVA + Fisher's vs. unstressed rats of the same sex (*), or vs. the other sex of the same group (unstressed or PRS rats) (#)).

Table 2 - Effect of early life stress and gender on steady-state monoamine levels in the nucleus accumbens (NAc) and prefrontal cortex (PFC).

	Monoamines (pg/mg)	CONT ♂	PRS ♂	CONT ♀	PRS ♀
Nucleus accumbens	NA	13.93 ± 2.45#	24.94 ± 6.05#	285 ± 62.1#	136 ± 17.7*#
	A	53.88 ± 11.9#	57.38 ± 9.14	176 ± 25.0#	81.5 ± 7.73*
	DA	19.55 ± 1.91#	47.71 ± 10.6	428 ± 115#	174 ± 15.2*
	5HT	157.8 ± 26.6#	56.33 ± 12.1#	348 ± 85.3#	189 ± 26.3*#
Prefrontal cortex	NA	19.92 ± 3.30	19.72 ± 2.39#	25.0 ± 3.40	37.2 ± 2.59*#
	A	26.13 ± 12.2	44.70 ± 15.7	29.1 ± 8.48	36.5 ± 1.45
	DA	40.22 ± 6.05	21.50 ± 3.51#	37.4 ± 5.14	61.6 ± 9.17*#
	5HT	74.48 ± 7.25#	43.24 ± 6.72	216 ± 52.7#	76.5 ± 14.2*

Data are means ± S.E.M of 4-5 determinations. CONT = control unstressed rats; NA = noradrenaline, A = adrenaline, DA = dopamine. $p<0.05$ (Two-way ANOVA + Fisher's vs. unstressed rats of the same sex (*), or vs. the other sex of the same group (unstressed or PRS rats) (#)).

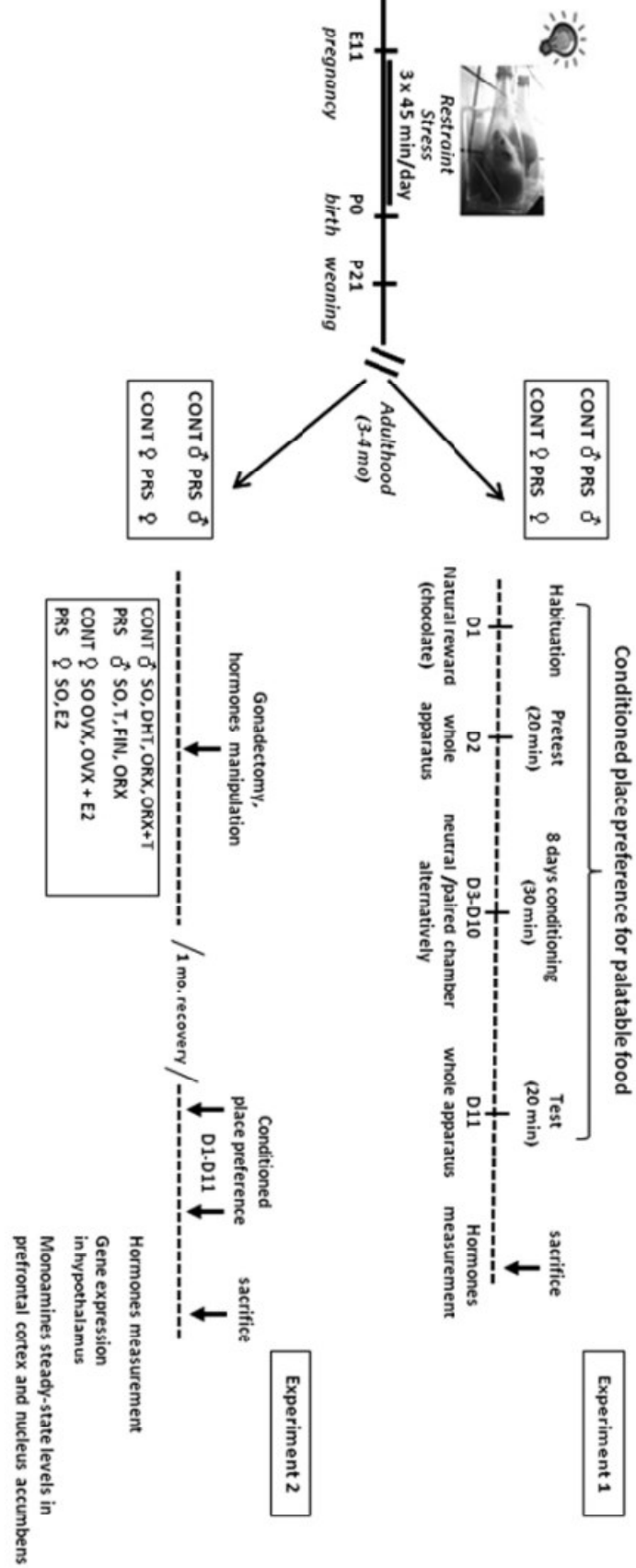


Fig. 1- Reynaert et al.

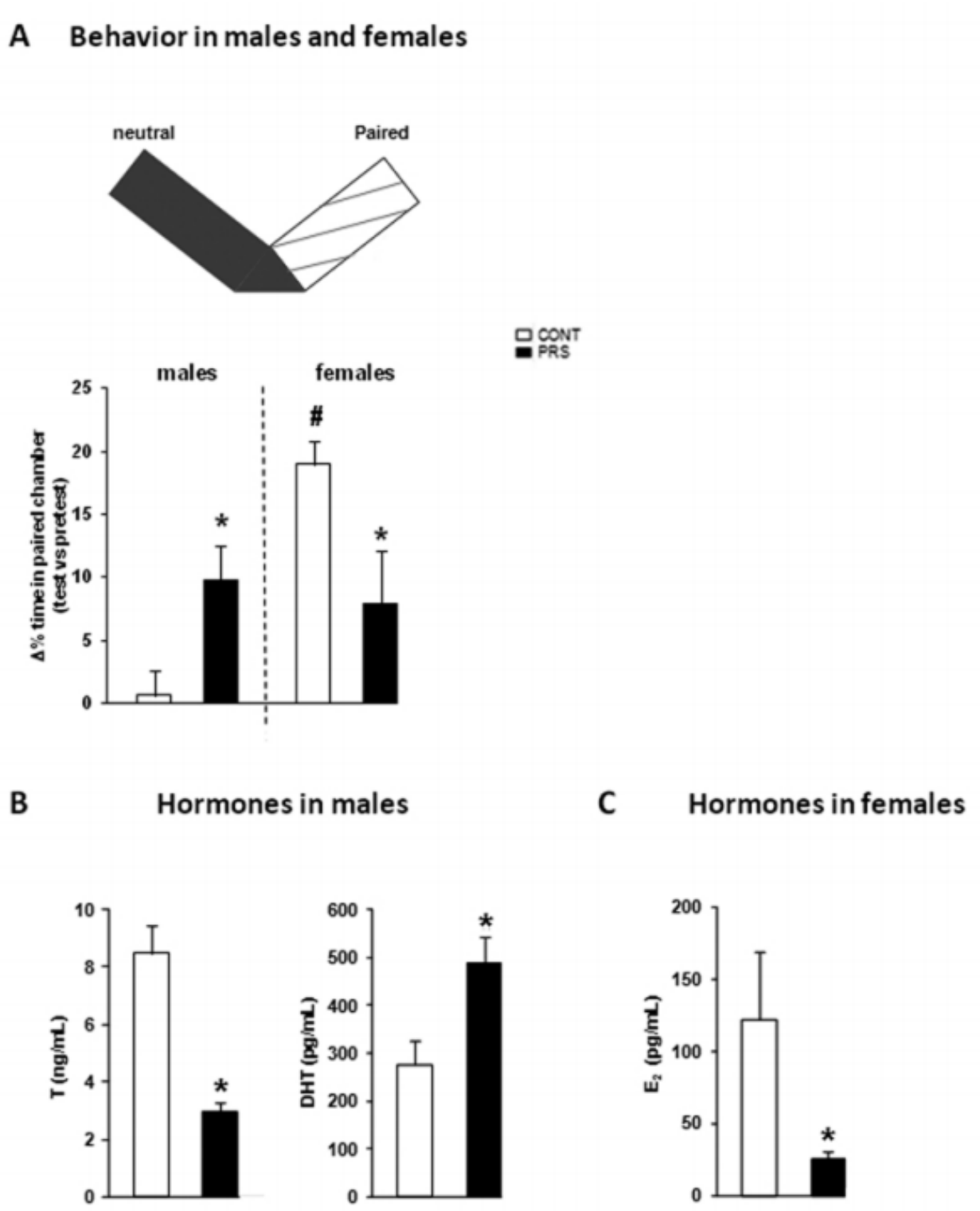
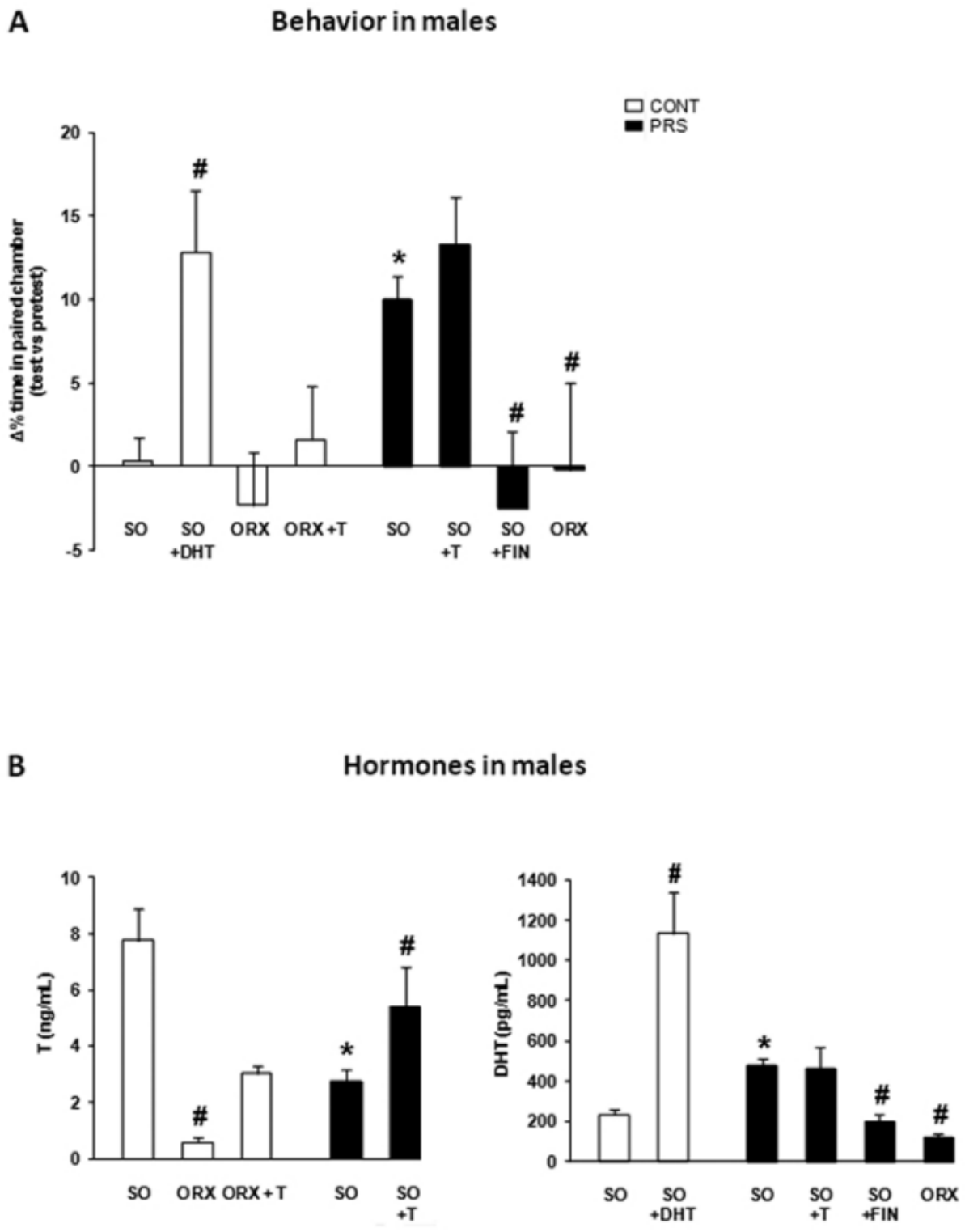
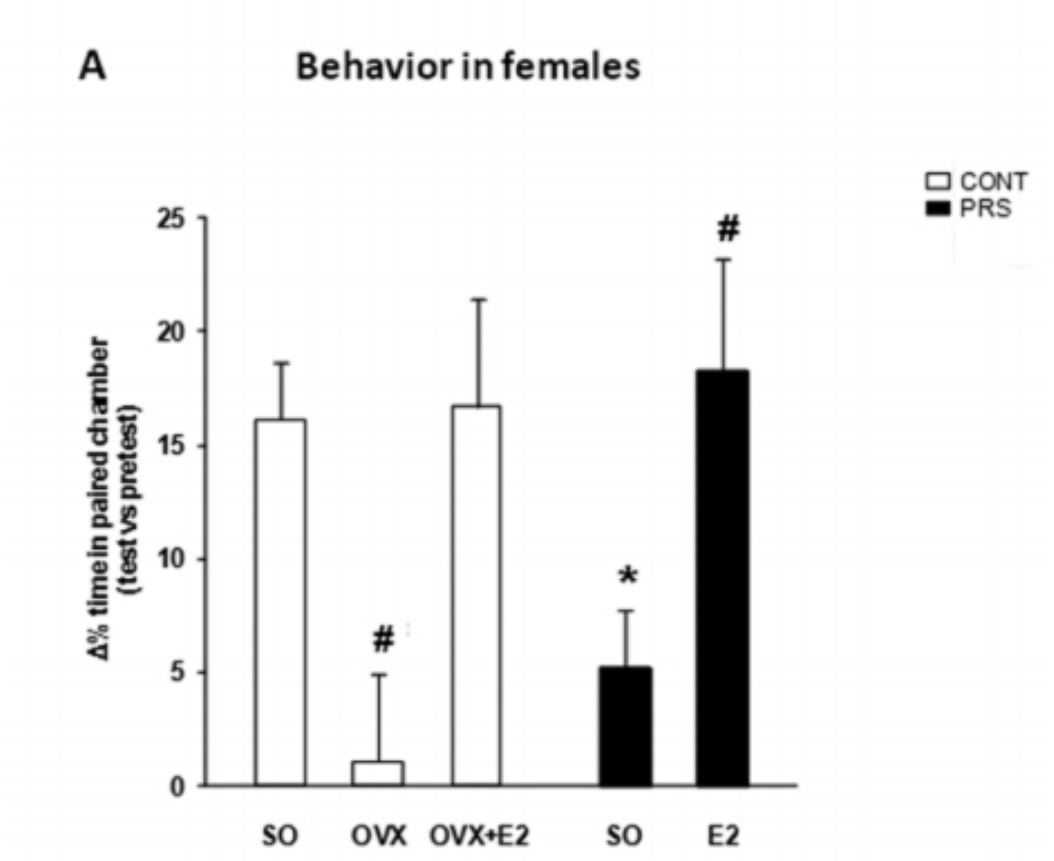
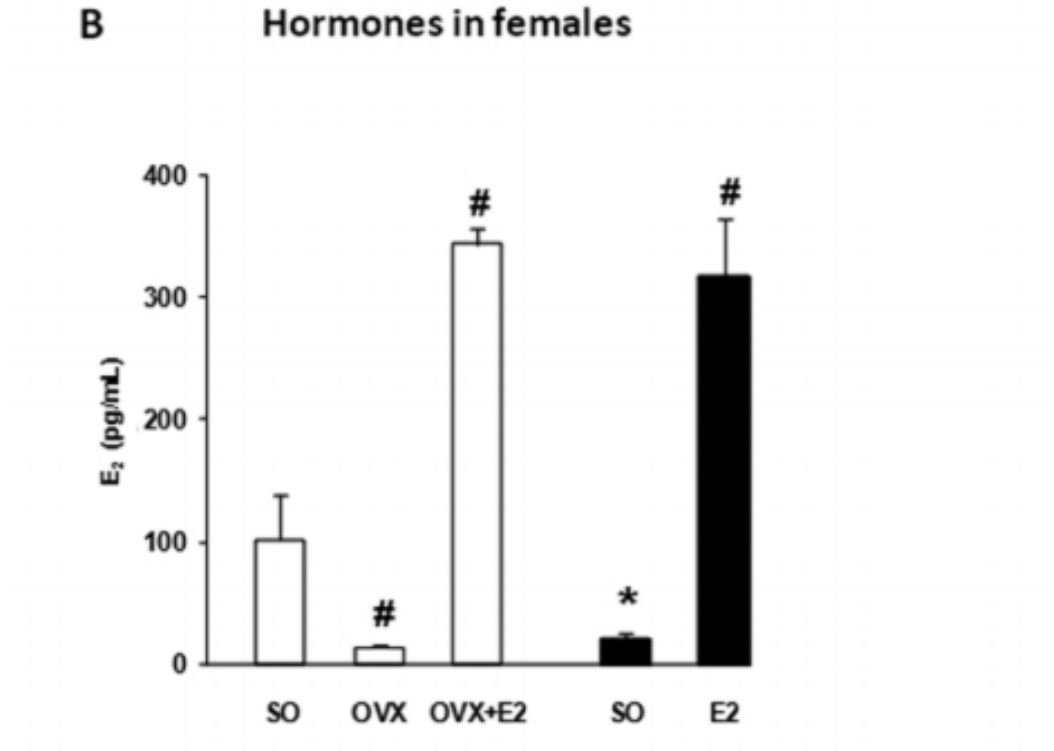
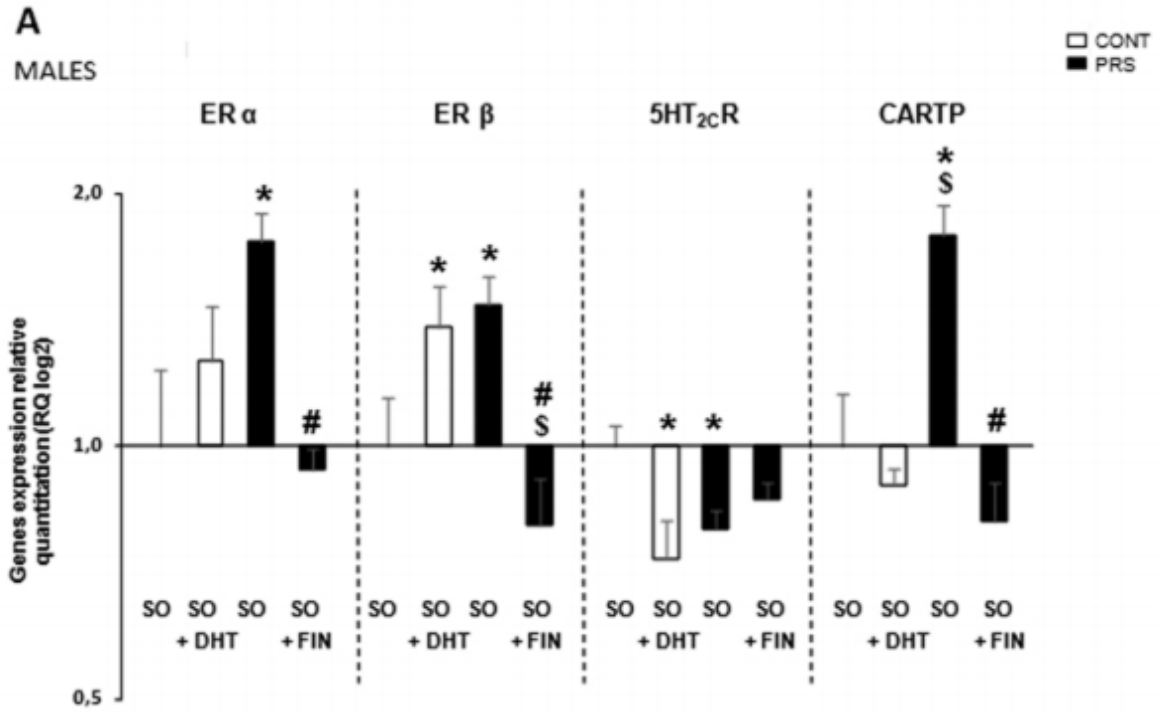


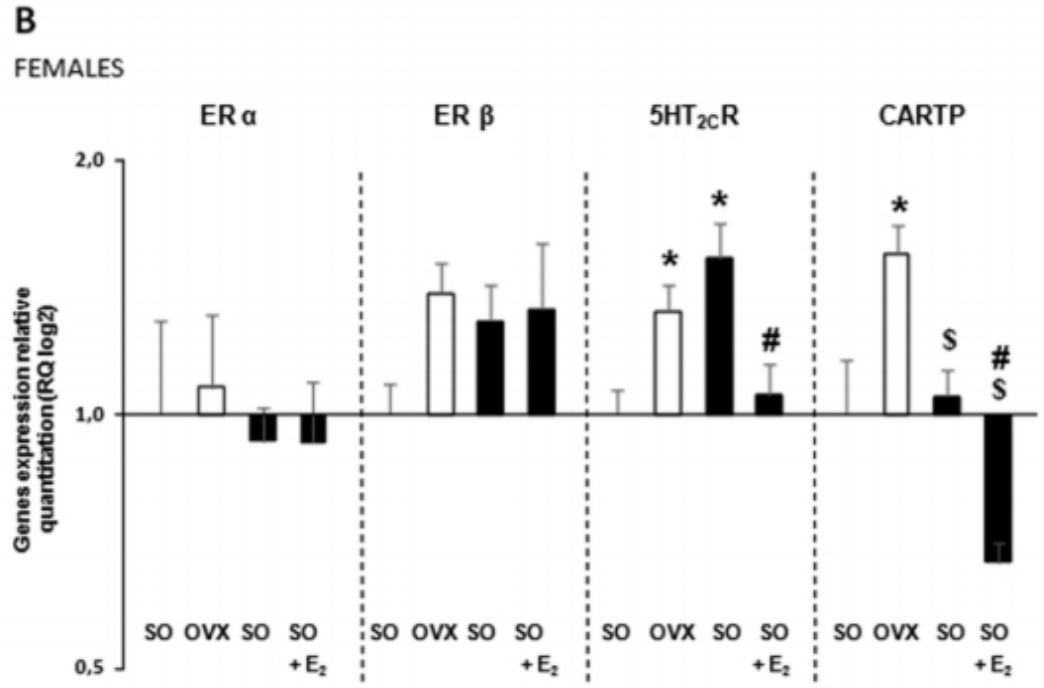
Fig. 2- Reynaert et al.

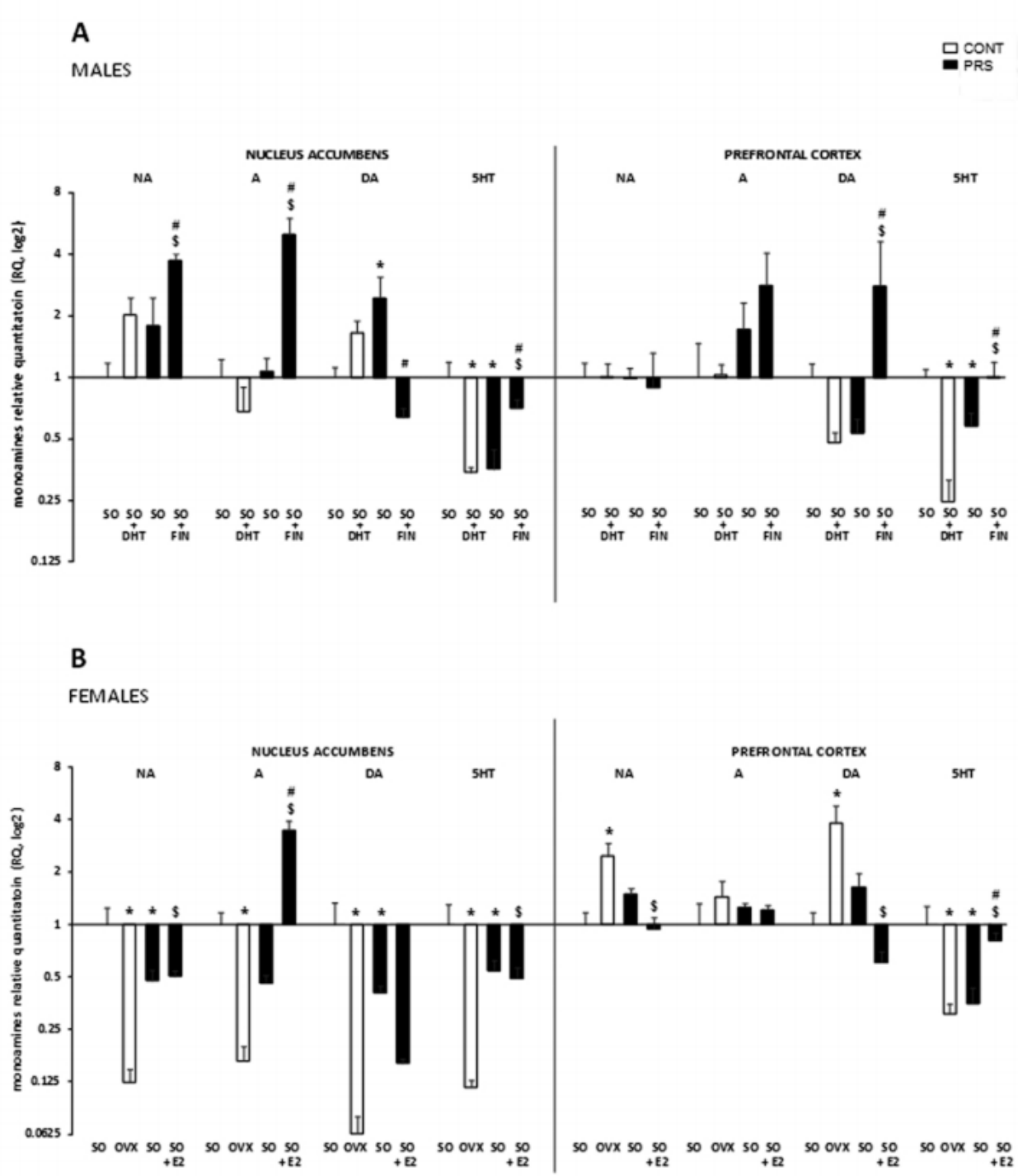












CHAPTER III: A SELF-MEDICATION HYPOTHESIS FOR ENHANCED PRS RESPONSE TO COCAINE AND SEX DIFFERENCES

5- Antidepressant-like effect of cocaine is associated with increased reward in male and female prenatally restraint stressed rats

Cocaine is known to induce depression and anxiety, when administered (Paine, Jackman and Olmstead, 2002), but mainly during withdrawal, even at early stage, 24-44 hours after last cocaine injection of a chronic sensitization paradigm (Rudoy and Van Bockstaele, 2007; De Oliveira Cito et al., 2012). Also, high anxiety predisposes to cocaine self-administration (Dilleen et al., 2012). However, very little is known concerning a putative beneficial effect of cocaine during chronic sensitization. In the introduction and chapters 1 and 2, we have shown that PRS animals display depressive-like behavior and enhanced anxiety in comparison to control unstressed animals. They also show an increased sensitiveness toward drugs of abuse, regarding active research behavior, drug-induced locomotor activity and preference for drug-paired chamber in CPP. An important sex effect was also demonstrated in the analyzed profiles. We have thus shown that the PRS model was a very valid model which program animals to develop both anxious-/depressive-like phenotype and addiction.

Here, we wanted to study these parameters as two inter-related pathologies, and we address the question of a putative anxiolytic/antidepressive effect of cocaine at the basis of the increased sensitiveness to drugs in PRS rats.

Preclinical evidence suggests that metabotropic glutamate (mGlu) receptors, which mediate glutamate neurotransmission, and are located throughout limbic and cortical brain sites, are implicated in drug addiction. mGlu receptors play a crucial role in regulating behavioral effects of drugs of abuse relevant to drug addiction. Specifically, antagonists at excitatory postsynaptic mGlu5 receptors decrease drug self-administration, while agonists at inhibitory presynaptic mGlu2/3 receptor agonists prevent reinstatement to drug-seeking and -taking after a period of abstinence (Markou, 2007). We thus analyzed the expression of these receptors in the NAc to identify neuropathological processes associated with cocaine administration.

Manuscript in preparation

Antidepressant-like effect of cocaine is associated with increased reward in male and female prenatally stressed rats

Marie-Line Reynaert[1,4], Eleonora Gatta[1,4], Jordan Marrocco[2], Jérôme Mairesse[1,4], Gilles Van Camp[1,4], Ferdinando Nicoletti[3,4], Sara Morley-Fletcher[1,4*], Stefania Maccari[1,4*]

*Co-last authors

[1]Neural Plasticity Team, UMR 8576/UGSF, CNRS/University Lille1, Lille, France
[2]IRCCS Centro Neurolesi "Bonino-Pulejo", Messina, Italy
[3]Dept of Pharmacology, Sapienza University of Rome and IRCCS Neuromed, Pozzilli, Italy
[4]LIA - International Associated Laboratory – Prenatal Stress and Neurodegenerative Diseases, University of Lille1/CNRS, Villeneuve d'Ascq, France; Neuromed, Pozzili and Sapienza University of Rome, Italy.

Address for correspondance:
Prof. Stefania Maccari, Ph.D., HDR
University Lille 1, France,
Co-Director LIA, France
Neural Plasticity Team – CNRS UMR 8576/ UGSF
Structural and Functional Glycobiology Unit
Bât C9, Avenue Mendeleiev –59655 Villeneuve d'Ascq France
Office: +33.32033.6042; Fax +33.32043.6555
e-mail : stefania.maccari@univ-lille1.fr

Abstract

Prenatal Restraint Stress (PRS) in rats is a well-documented model of early stress known to induce long-lasting neurobiological and behavioral alterations as increased sensitiveness to psychostimulants, anxiety/depression-like behavior and impairment in the glutamate machinery and metabotropic glutamate receptors system. Thus, the epigenetic programming modulated by early life stress predisposes to drug abuse disorders and anxiety/depression like behavior in the same animal model. We aimed to address whether a behavioral sensitizing history of cocaine could have a beneficial impact on the anxious/depressive phenotype in PRS rats. Since the anxiety-like profile of PRS rats is sex-dependent, with PRS male rats being anxious, while the depression-like profile appears to be characteristic of both sexes, we examined both males and female PRS adult rats in the response to cocaine. We report that PRS enhanced conditioned place preference for cocaine in both sexes with respect to unstressed rats. Remarkably, cocaine exerted an anxiolytic effect in PRS males and had anti-depressive properties in both males and female PRS rats. Interestingly, the reversal of the PRS anxious/depressive like behavioral phenotype induced by chronic cocaine treatment was associated with increased mGlu 5 receptors levels in males and mGlu 2/3 in both males and females in the nucleus accumbens. Our findings indicate drug addiction and anxious/depression as stress-related interdependent disorders in the PRS model in males and females, where the enhanced response to cocaine would be a self-medication strategy to counteract the anxious/depressive like phenotype induced by PRS. Changes occurring in metabotropic glutamate receptors system in the nucleus accumbens and in the hippocampus would be the shared common neurobiology of the pathological programming that alters the activity of the reward circuit in response to prenatal stress both as a model for depression- and addiction-like disorders.

Keywords: prenatal stress, cocaine, conditioned place preference, anxiety, depression, nucleus accumbens, metabotropic glutamate receptors

Introduction

People who have experienced stressful/traumatic situations display high prevalence of anxious-depressive and addictive disorders (Keyes et al., 2012). Growing evidence suggests mental illness and addiction vulnerability co-emerge from a unified etiology with shared environmental and genetic factors (Chambers et al., 2001, 2010a; Brewer et al., 2010) and new literature show that alterations in reward and motivational processes may constitute the defining characteristics of both depression and addiction (Russo & Nestler, 2013). At neurocircuitry level, abnormalities in the frontal cortex and hippocampus also span mental disorders frequently occurring with addictions and anxious depression (Chambers et al., 2010b, 2013). Among substance abusers with new admissions in clinics, almost a third is taking antidepressants. Women often develop the mood disorder first while men frequently develop such disorders after the addiction, thus indicating that sex differences shape this comorbidity, too. Nevertheless, it is not clear if drug dependence and depression are different behavioral expressions of the same neurobiological abnormalities, or whether one psychiatric disorder leads the other (Markou et al., 1998).

Stress, and in particular early life stress events, modulates the activity of the reward neuronal circuit, thus constituting a risk factor for anxious/depressive and addictive disorders (Charmandary et al., 2003; Seckl et al., 2008). In adult “PRS rats”, i.e. the offspring of dams exposed to repeated episodes of restraint stress (PRS: prenatal restraint stress) during pregnancy, the pathological epigenetic programming triggered by early life stress predisposes to both drug abuse disorders and anxiety/depression-like behaviors. PRS rats display an anxious/depressive like phenotype (Maccari et al., 1995; Dugovic et al., 1999; Darnaudery et al., 2006; Maccari and Morley-Fletcher, 2007). PRS rats show neurochemical alterations, including glutamate and metabotropic glutamate receptors, and circadian rhythm abnormalities that are indicative of an anxious/depressive phenotype (Dugovic et al., 1999; Morley-Fletcher et al., 2011; Marrocco et al., 2012; Laloux et al., 2012; Mairesse et al., 2013; Marrocco et al., 2014). PRS rats also show an enhanced vulnerability toward addiction (Deminière et al., 1992; Koehl et al., 2000; Morley-Fletcher et al., 2004; Van Waes et al., 2009; Kippin et al., 2008).

While several studies conducted on PRS rats have evidenced functional alterations in dopamine system in the nucleus accumbens associated to enhanced drug use (Alonso et al., 1994; Henry et al., 1995; Rodrigues et al., 2012; Baier et al., 2012), we have proven increasing evidence that alterations in group-I and group-II metabotropic glutamate receptors

and glutamatergic transmission participate to the anxious/depressive profile induced by PRS (Zuena et al., 2008; Van Waes et al., 2009, 2011; Laloux et al., 2012; Marrocco et al., 2012, 2014). Indeed, antidepressants correct the PRS phenotype by restoring metabotropic glutamate (mGlu) receptors system (Morley-Fletcher et al., 2011) as well as glutamate transmission in the ventral hippocampus (Marrocco et al., 2014). Evidence is accumulating that mGlu5 receptors play an important role in regulating the reinforcing and incentive motivational properties of drugs of abuse (Kenny et al., 2005; Paterson & Markou, 2005). Indeed, mGlu5 receptor antagonists may be useful in decreasing the reinforcing effects of psycostimulants and preventing relapse during protracted abstinence, whereas mGlu 2/3 receptor antagonists may alleviate the depression observed during the early nicotine withdrawal phase (Markou et al., 2007). Thus, neuroplastic changes occurring in the glutamate machinery in the PRS brain may lie at the core of the pathological programming that alters the activity of the reward circuit that would lead either to a depressive or addictive phenotype in response to prenatal stress. At present, the influence of PRS on mGlu receptors system in the nucleus accumbens is unknown.

Some of the hallmarks of PRS rats, such as anxiety-like behaviour and changes in hippocampal neuroplasticity, are gender-dependent (Zuena et al., 2008; Darnaudery & Maccari, 2008), whereas others, such as depression-like behaviour and response to natural reward, are not (Koehl et al., 1999; Van Waes et al., 2011; Reynaert et al., 2012). Moving from previous evidence that both male and female PRS rats display cognitive and depressive-like behavior that can be prevented by ethanol consumption during adolescence (Van Waes et al., 2009, 2011), we addressed whether a behavioral sensitizing history of cocaine could have a beneficial impact on the anxious/depressive phenotype and on the mGlu receptors system in PRS males and female rats.

Methods

Animals

Adult female Sprague-Dawley rats weighing about 250 g and sexually experienced males (400-500 g) were purchased from Charles River Laboratories (L'Arbresle Cedex, France). Females were group-housed for 3 weeks for acclimation and oestrous cycle coordination in a temperature (22+/-2°C) and humidity-controlled room under a 12h light-dark cycle with lights off at 20h. Males were single-housed during this period. Water and chow were provided *ad*

libitum. Then, females were placed with a male for a night and the day corresponding to spermatozoids revealing by microscopy or copulation plug visualisation was designated as embryonic day 0 (E0), and females were housed individually in transparent Plexiglas cages and randomly assigned to control or stressed group (n=14 per group).

Prenatal restraint stress procedure

Pregnant females were subjected to restraint stress according to our standard protocol (Maccari et al., 1995; Morley-Fletcher et al., 2003). At E11 of pregnancy until delivery, female rats were submitted to three stress sessions daily (45 min each), during which they were placed in transparent plastic cylinders and exposed to bright light or were left undisturbed (control dams).

Only rats from litters of 10-14 rats with a similar number of males and females were used. After weaning (P21), offspring grew up until reaching the good stage (adulthood) to be used for experimentation (2-3 brothers/2-3 sisters per cage). All experiments followed the rules of the European Communities Council Directive 86/609/EEC. The local ethic committee approved the prenatal stress procedure.

Drug administration

Cocaine (cocaine hydrochloride, Sigma-Aldrich, France) was prepared extemporaneously in saline solution (NaCl 0.9%) and administered intraperitoneally (i.p.). For behavioral sensitization, escalating doses of the drug were administered for 10 days (15 mg/mL/kg for 2 days, 20 mg/mL/kg for 3 days and 30 mg/mL/kg for 10 days). The time line of experiments is depicted in figure 1.

Assessment of anxiety-like behavior

Light and dark test. On day 7 of the chronic sensitization procedure, rats (n=7-8 per group) were injected with vehicle or cocaine (30 mg/mL/kg) and anxiety-like behavior was assessed as previously described (Marrocco et al., 2014). The light and dark box setup consisted of two compartments: one light compartment (45 × 32 × 32 cm, 50 lux; light box) and one dark compartment (30 × 32 × 32 cm, 5 lux; dark box). The compartments were connected via a small opening (10 × 15 cm) enabling transition between the two boxes. Rats were placed in the light compartment and the time spent in each compartment and the latency to the first entry into the light compartment during the 5 min test, were assessed on-line *via* a video camera located above the box. Behavior was automatically analyzed using video tracking software (View Point).

Assessment of depressive-like behavior

Forced swim test. At day 9 and 10 of the chronic cocaine treatment, rats (n=7-8 per group) were injected with vehicle or cocaine (30 mg/mL/kg) and subjected to an adapted version of the forced swim test (Porsolt et al. 1978) as previously described (Marrocco et al. 2014). Following injection, control and PRS male and female rats were placed in a cylindrical container (height, 59 cm; diameter, 25 cm) filled with water at 25°C up to a level of 36 cm. The test was performed between 12:00 and 5:00 P.M. Twenty-four hours after a 15 min session (pretest, on day 9), rats were tested (day 10) during a 5 min session, during which immobility latency and duration, climbing, and swimming were automatically analyzed using video-tracking software (View Point).

Conditioned Place Preference (CPP)

Saline solution (Nacl 0.9% in distilled water) was used as vehicle neutral stimulus, whereas cocaine (15 mg/mL/kg) was used as rewarding stimulus. To evaluate the influence of PRS and sex differences on preference to cocaine-paired chamber, a separate set of adult (4 mo old) male and female control and PRS rats (n=7-8 rats per group) was used for the experiment. The CPP apparatus was made in opaque Plexiglas and had two compartments with different associated visual cues (one chamber white and the other grey). On day 1 (pretest), rats were allowed to explore the whole apparatus for 20 minutes in the absence of any stimulus, in order to determine their spontaneous preference for one chamber of the apparatus. Conditioning (8 days, 30 min/session) with reward was conducted in the least preferred side. During conditioning, animals were alternatively injected with vehicle or cocaine just before being placed in the appropriate stimulus-paired chamber. Day 10 (test) was performed as pretest. Data were expressed as a difference of percentage of time spent in cocaine-paired chamber during the test minus the pretest.

Western blot analysis

Control and PRS rats (n=4 per group) were killed by decapitation, and nucleus accumbens was rapidly dissected and immediately stored at -80°C. Immunoblotting analysis was performed on the synaptosomes isolated from the NAc as previously described (Marrocco et al., 2014). To isolate synaptosomes, tissue was manually homogenized with a potter in 10 volumes of HEPES-buffered sucrose (0.32Msucrose, 4 mMHEPES pH 7.4). All procedures were performed at 4°C. Homogenates were centrifuged at 1000 x g for 10 min, and the

resulting supernatants were centrifuged at 10,000 x g for 15 min. The pellet obtained from the second centrifugation was resuspended in 10 volumes of HEPES-buffered sucrose and then spun again at 10,000 x g for 15 min. This pellet contained the crude synaptosomal fraction. BCA assay was used to determine protein concen-tration. Synaptosome lysates were resuspended in Laemmli reducing buffer, and 3 µg of each sample was first separated by electrophoresis on Criterion TGX 4 –15% precast SDS-PAGE gels (26 wells; Bio-Rad) and later transferred to nitrocellulose membranes (Bio-Rad). Transfer was performed at 4°C in a buffer containing 35 mM Tris, 192 mM glycine, and 20% methanol. We used the following primary antibodies: rabbit polyclonal anti-mGluR2/3 (1:500; Upstate, catalog # 06-676), rabbit polyclonal anti-mGluR5 (1:500; Millipore, AB5675), and mouse anti β-actin (1:20000; Sigma, catalog #A5316). All primary antibodies were incubated overnight at 4°C. HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (purchased from GE Healthcare) were used at a dilution of 1:10000 and were incubated for 1 h at room temperature. Densitometric analysis was performed with Quantity One software (Bio-Rad) associated with a GS-800 scanner. The ratio of individual proteins to β-actin was then determined, and these values were compared for statistical significance.

Statistical analysis

Data were analyzed by two-way ANOVA for CPP (group by sex) or three-way ANOVA (group by sex by treatment) for other behavioral test and western blots. The Fisher's post hoc test was used to isolate the differences. A p value <0.05 was considered to be statistically significant.

Results

Cocaine reversed the anxious and depressive-like phenotype of PRS rats

Anxiety-like behavior is shown in **Fig. 2.** Chronic treatment with cocaine (7 days) exerted an anxiolytic effect on PRS male rats and unstressed control female rats, namely, rats which display an anxious-like phenotype under basal conditions (treated with vehicle) (Time in white, ANOVA, sex x group x treatment, $F_{(1.48)}= 8.82$, $p<0.01$; latency to enter the white box, sex x group x treatment, $F_{(1.48)}= 7.45$, $p<0.01$).

Depressive-like behavior is shown in **Fig. 3**. Chronic treatment with cocaine reduced immobility behavior displayed by PRS rats in the forced-swim test (ANOVA, group x treatment, $F_{(1.49)}= 4.63$, $p<0.05$).

PRS enhanced preference for cocaine in both male and female rats

We examined the behavior of adult PRS and control unstressed male and female rats in the conditioned place preference for cocaine. We found an enhanced preference for cocaine-paired chamber both in male and female PRS rats (ANOVA, group effect, $F_{(1,28)}=9.02$, $p<0.01$, sex effect, $F_{(1,28)}=10.03$, $p<0.01$, **Fig. 4**).

Cocaine enhanced metabotropic glutamate receptors levels in the NAc of PRS rats

PRS had no effect on the expression of mGlu 5 and mGlu2/3 receptors in the NAc while chronic cocaine treatment increased the expression of mGlu5 and mGlu 2/3 receptors levels in PRS animals (**Fig 5).** In particular, chronic cocaine increased mGlu5 in PRS males and in control unstressed females (ANOVA, sex x treatment, $F(1,24)=5.132$, $p< 0.05$) namely, the animal which displayed anxious behavior in the light dark test, while it increased mGlu 2/3 in both PRS males and females (ANOVA, group x treatment ($F1,24)=4.830$, $p< 0.05$). Thus, the enhanced expression of mGlu 5 and mGlu 2/3 in the NAc was in accordance with to the anxiolytic action exerted by cocaine in PRS males and the antidepressant effect in PRS males and females.

Discussion

Here, we wanted to assess a putative beneficial effect of cocaine on anxious-depressive like symptoms in the enhanced sensitiveness observed in PRS rats. We report that cocaine exerted an anxiolytic effect in PRS males and had anti-depressive properties in both males and female PRS rats. PRS enhanced conditioned place preference for cocaine in both sexes with respect to unstressed rats. The reversal of the PRS anxious/depressive-like behavioral phenotype induced by chronic cocaine treatment and cocaine-induced CPP was associated to increased mGlu5 receptors levels in males and increased mGlu 2/3 receptors in both males and females in the NAc.

At present, most of research on anxiety and depression-like symptoms associated with cocaine use has been conducted on cocaine withdrawal syndrome, which is characterized by greater cocaine withdrawal-induced anxiety-like behavior and anhedonic depression-like mood (Markou & Koob, 1991). To our knowledge, this is the first study that investigates the effects of cocaine as an antidepressant or anxiolytic drug on mood disorders-associated behavior during the chronic treatment window, and in an animal model of early life stress. Remarkably, cocaine reversed anxiety and depression-like behavior in PRS rats, thus acting as a disease-dependent drug. Indeed, its anxiolytic action in the light /dark test was exerted only on PRS males which are more anxious, and additionally on unstressed control females (which are more anxious than PRS females).

Neurochemically, an increase in the levels of mGlu 5 and mGlu 2/3 in the NAc followed chronic administration of cocaine and was associated to behavioral changes in PRS rats.

This profile is consistent with reports of increased glutamatergic transmission in brain structures involved in the regulation of reward processes following administration of psychostimulants, such as the dorsal striatum (McKee & Meshul, 2005), NAc (Pierce et al., 1996) and ventral tegmental area (Kalivas & Duffy, 1995). Moreover, drug-induced adaptations in glutamatergic neurotransmission have been suggested to be involved in the development of drug dependence (Kalivas et al., 2005; Kenny et al., 2005).

PRS rats are characterized by reduced levels of mGlu5 for males and mGlu2/3 for males and females in the hippocampus (Zuena et al., 2008), a profile which is consistent with the sex dimorphic effect of PRS on anxiety but not on depression, and the main involvement of mGlu5 in anxiety and mGlu2/3 in depression (Nicoletti et al., 1996, 2005). At present, the influence of PRS on mGlu has been measured only in the hippocampus region (Zuena et al., 2008; Van Waes et al., 2009, 2011; Morley-Fletcher et al., 2011). Interestingly, in the NAc,

PRS did not affect mGlu 5 or mGlu 2/3 levels whereas cocaine increased it. Cocaine enhanced mGlu receptors in accordance with reversal of anxious/depressive behavior, with a selective action on mGlu5 in more anxious PRS male rats. Alterations of glutamatergic transmission induced by PRS occur early in development, since it is possible to detect lower levels of mGlu5 at pnd14 and of mGlu2/3 at pnd 22 (Laloux et al., 2012).

Most of neuroadaptive changes found in PRS rats occur in the ventral hippocampus (Zuena et al., 2008; Marrocco et al., 2012, 2014). The ventral hippocampus critically regulates the firing rate of dopaminergic neurons in the ventral tegmental area, which project to the shell of NAc and mediate drug-seeking behavior (Patton et al., 2013). Early changes in glutamatergic transmission and mGlu receptors system could participate in to the set up of the reward system. In support of this hypothesis, it has been shown that the mesencefalic afferents to the Nac present a dual DA-glutamate phenotype (Dal Bo et al., 2004, 2008; Berger et al., 2002). Indeed an induction or de-repression of the glutamatergic phenotype of DA neurons has been reported following a neonatal injury (Dal Bo et al., 2008). The expression of glutamatergic phenotype in DA neurons may be a regulated phenomenon that might be re-programmed by prenatal insult such as prenatal stress. Recent findings demonstrate that acute cocaine inhibits DA and glutamate release from midbrain DA neurons via presynaptic D2R but has differential overall effects on their transmissions in the NAc (Adrover et al., 2014). Cocaine, by blocking DA reuptake, would prolong DA transients and facilitate the feedback inhibition of DA and glutamate release from these terminals (Adrover et al., 2014).

Although much study is still needed to unravel the mechanisms of glutamate-DA interaction and its long-term vulnerability to gestational stress, it is clear that PRS induces a disblanced function of both neurotransmitter pathways (Henry et al., 1995; Berger et al., 2002; Zuena et al., 2008; Marrocco et al., 2012). Such imbalance might be inter-regulated in a regional basis at the level of NAc and ventral hippocampus and shape PRS dual anxious /depressive – addicted phenotype.

Our findings lay the groundwork for the study of the influence on early life stress on drug addiction as an interdependent and secondary symptom of anxious/depression phenotype in the PRS model. As a whole, the existence of comorbidity in depression and drug abuse, underlines the importance of the adoption of an integrated approach in the treatment of these disorders, where the brain reward system could be considered also as a potentially important therapeutic target for anxiety and depression. An elucidation of the neurobiological and behavioral mechanisms mediating this comorbidity would not only lead to the development of better treatments for these two psychiatric disorders, but would also enhance our

understanding of the mechanisms sub-serving motivational and affective processes in both healthy and diseased individuals. Thus, it would be interesting to explore hypothesis generated in the field of depression in animal models of drug dependence and vice versa. Furthermore, exploration of the self-medication hypothesis should be aimed at testing whether various drugs of abuse reverse depressive symptomatology in animal models of depression.

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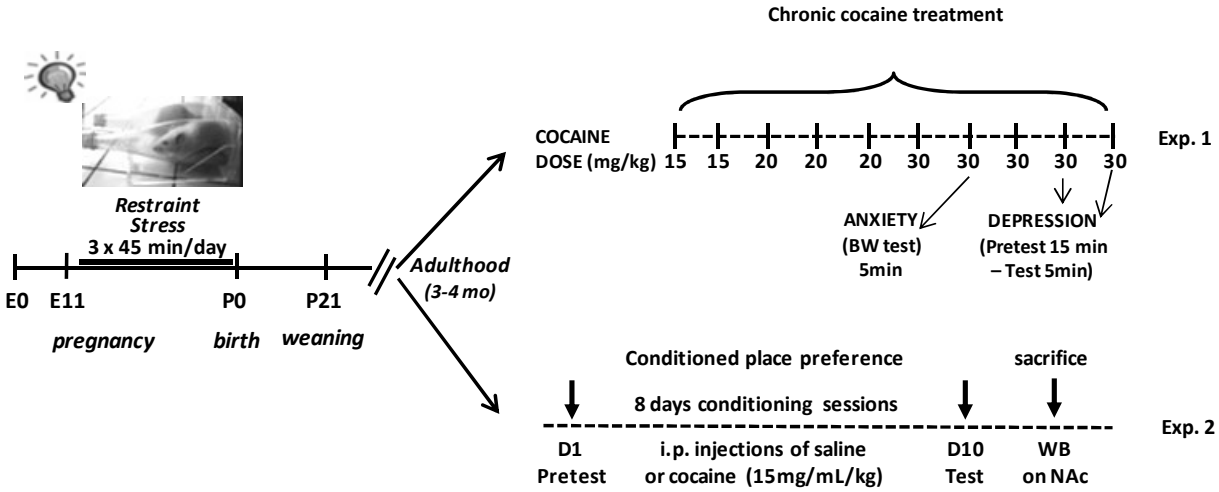

Fig. 1 Reynaert et al

Experimental time line Adult PRS and CONT unstressed male and female rats (n=7-8 rats per group) were injected daily i.p. with escalating doses of cocaine 15 -30 mg/kg). On day 7 of chronic cocaine treatment, rats were assessed for anxious-like behavior in the light and dark test. On days 9 and 10, depressive-like behavior was measured in the forced-swim test. In a second experiment, a separate set of PRS and CONT animals was tested for preference to cocaine in a conditioned place preference paradigm. 48 hours after the test, rats were killed and brains dissected for analysis of mGlu 5 and mGlu2/3 receptors in the western blot (WB) in nucleus accumbens (NAc).

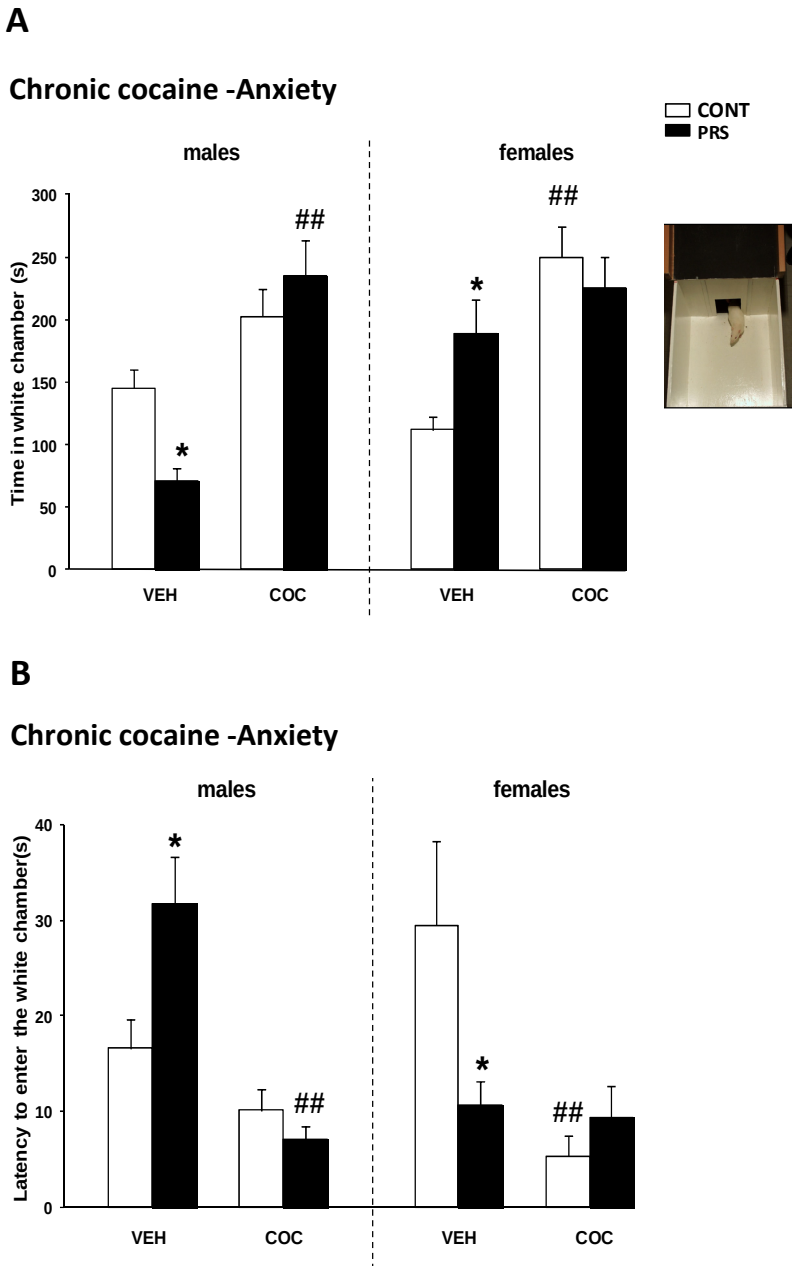

Fig. 2 Reynaert et al.

Cocaine exerted an anxiolytic effect in PRS male rats On day 7 of chronic cocaine treatment, male and female control unstressed and PRS rats are injected with cocaine (30 mg/mL/kg, i.p.) or vehicle and tested for their anxious-like profile in the light and dark box. The experiments take place between 2:00 and 5:00 PM. The time spent (s) in the white chamber and the latency to visit the white chamber are represented, respectively in (A) and (B). Values are means $\pm$ SEM of 7-8 rats per group, *p<0.05 vs control animals, ## p<0.01 vs vehicle-treated animals of the same group (Control or PRS) and sex. CONT=control unstressed rats, VEH=vehicle-treated, COC=cocaine-treated.

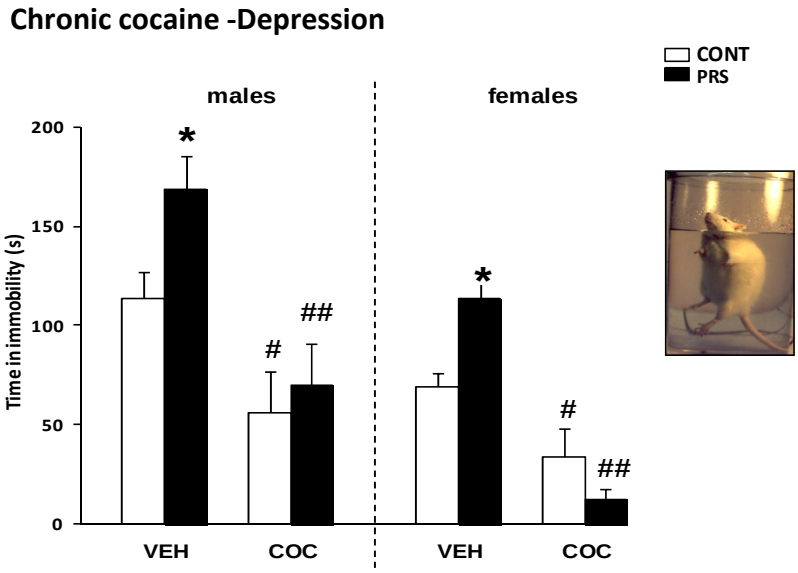

Fig. 3 Reynaert et al.

Cocaine had antidepressant properties in PRS male and female rats On day 9 (prestest, 15 min) and 10 (test, 5 min) of chronic cocaine treatment, male and female control unstressed and PRS rats are injected with cocaine (30 mg/mL/kg, i.p.) or vehicle and tested for their depressive-like profile in the light and dark box. The experiments take place between 2:00 and 5:00 PM. The time spent in immobility during the test is represented. Values are means $\pm$ SEM of 7-8 rats per group, *$p<0.05$ vs control animals, #$p<0.05$ vs vehicle-treated animals of the same group (Control or PRS) and sex, ## $p<0.01$ vs vehicle-treated animals of the same group (Control or PRS) and sex. CONT=control unstressed rats, VEH=vehicle-treated, COC=cocaine-treated.

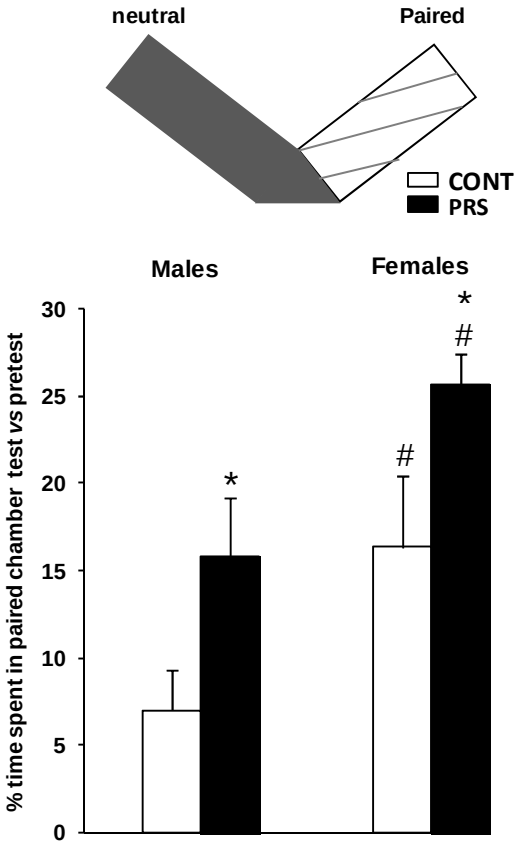

Fig. 4 Reynaert et al.

Cocaine-induced conditioned place preference is increased in PRS rats. In experiment 2, male and female control unstressed and PRS rats were submitted to a conditioned place preference paradigm where they were injected alternatively with vehicle or cocaine (15 mg/mL/kg, i.p.) in vehicle- or cocaine-paired chamber for 8 days of conditioning sessions. Data are expressed as the percentage of time spent in the cocaine-paired chamber during the test versus the pretest. Values are means $\pm$ SEM of 7-8 rats per group, *$p<0.05$ vs control animals, #$p<0.05$ vs vehicle-treated animals of the same group (Control or PRS) and sex. CONT=control unstressed rats.

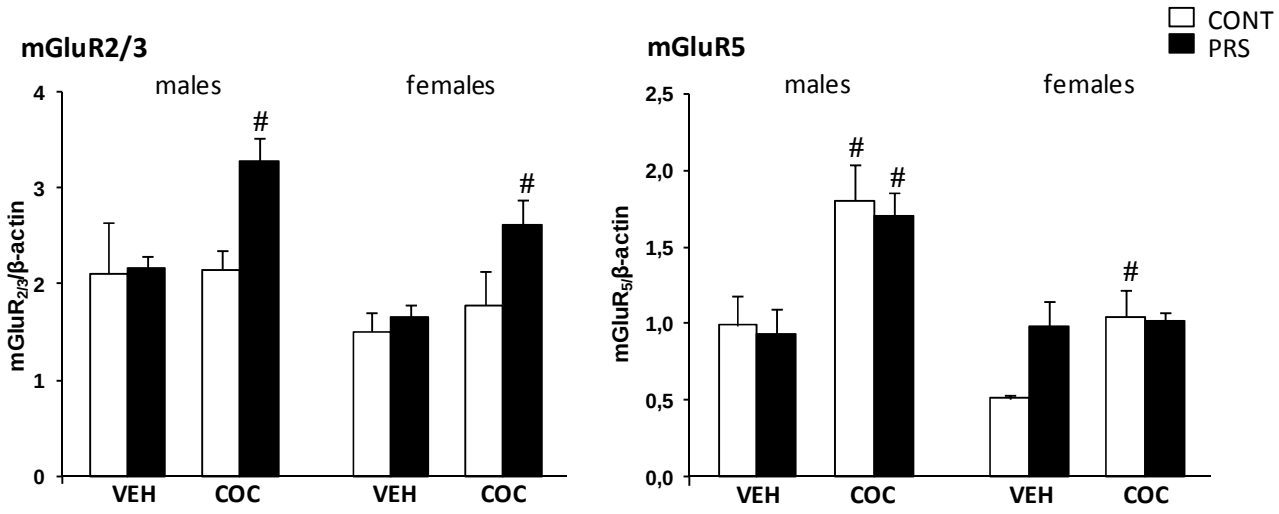

Fig. 5. Reynaert et al.

Cocaine-induced changes in the expression of mGlu receptors in Control and PRS male and female rats. After cocaine conditioned place preference (experiment 2), male and female control unstressed and PRS rats were are sacrificed and nucleus accumbens dissected. mGluR2/3 and mGluR5 expression are analysed by western blot. Data are expressed as values are means ± SEM of 4 rats per group, #p<0.05 vs vehicle-treated animals of the same group (Control or PRS) and sex. CONT=control unstressed rats, VEH=vehicle-treated, COC=cocaine-treated.

Conclusion – Cocaine effect on anxious-like profile of rats with modified hormonal status

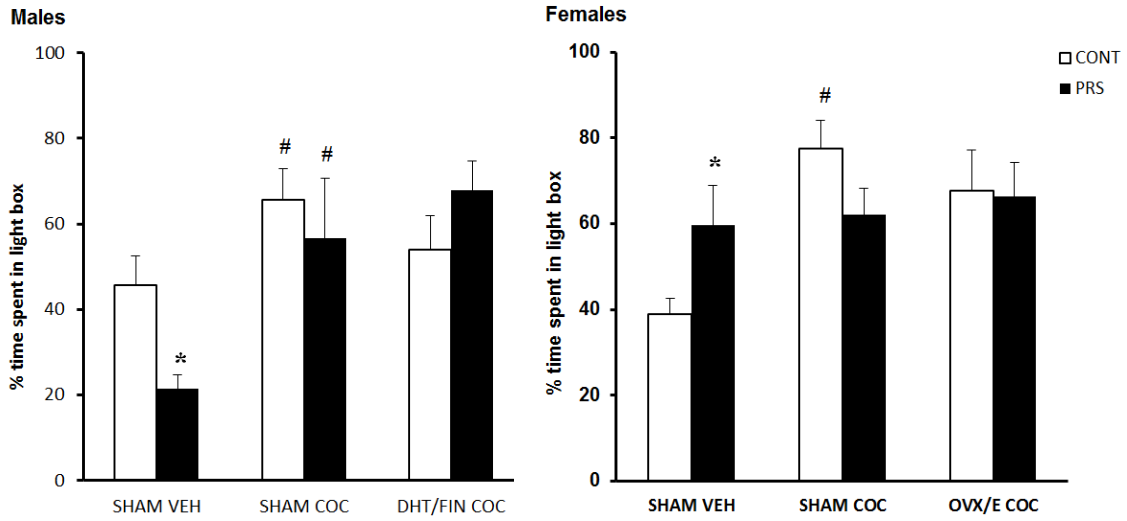

Fig 18 - Effect of cocaine in anxiety-like profile of rats hormonally modulated.

The effect of cocaine on the anxious-like profile was assessed in the light and dark test. One month after surgeries and hormonal treatment, rats (n=7-8 rats per group) received repeated injections of escalating doses of cocaine (from 15 mg/kg to 30 mg/kg i.p.). On day 7 of the chronic treatment, we assessed whether cocaine could modify the anxiety-like profile of rats with hormonal modification. Results are represented as the mean $\pm$ S.E.M of the percentage of time spent in the light chamber. CONT= control unstressed rats, SHAM=sham-operated animals, DHT=dihydrotestosterone supplemented, FIN=finasteride-supplemented, OVX=ovariectomy, E=estradiol-supplemented, VEH=vehicle, COC= cocaine. *p<0.05 vs. Control SHAM animals, #p<0.05 vs SHAM animals of the same group (CONT or PRS).

DHT-treated control unstressed rats, which display an anxious-like profile (Chapter 1) where not different from control animals after treatment with chronic injections of cocaine, showing, again, the anxiolytic effect of cocaine. The same beneficial effect of cocaine is observed in ovariectomized control females, that displayed an anxious-like profile before chronic administration of cocaine (Chapter one).

DISCUSSION

In the present thesis, we have shown that drug addiction and anxiety/depression are stress-related interdependent disorders in the PRS model, where sex hormones and glutamate lie at the core of this comorbidity.

1) Antidepressants are effective in PRS rats *via* a selective action of glutamatergic transmission

PRS-induced depressive-like profile, associated with impairment in glutamate machinery in the ventral hippocampus could be corrected by chronic treatment with the antidepressant (ATD) drug agomelatine, a 5-HT_{2C} receptor antagonist/melatonergic receptor (MT1/MT2) agonist, and at a lesser extent with the classical selective serotonin reuptake inhibitor (SSRI) fluoxetine. Abnormalities of glutamate release, with emerging evidence of glutamate role in the pathophysiology of depressive disorders (Hashimoto, 2009; Chaki et al., 2013), were coupled with large reductions in the levels of synaptic vesicle-associated proteins (SVP) (Marrocco et al., 2012). SVP are involved in the pathophysiology of depression (Tordera et al., 2007), and are sensitive to ATD treatment (Yamada et al., 2002; Rapp et al., 2004). The beneficial action of ATDs could thus pass through the modulation of SVP. In most cases, works have been carried out in control rats, or rats submitted to acute or chronic stress and have shown that ATDs aimed to reduce SVP and glutamate release, thereby concluding that an increase in glutamate release was responsible for the depressed phenotype (Bonanno et al., 2005; Musazzi et al., 2010; Tardito et al., 2010; Dagyte et al., 2011; Piroli et al., 2013; Milanese et al., 2013). However, selective changes in the expression of some mGlu receptors were found under basal or early-life stress conditions (O'Connor et al., 2013).

Here, we report that both fluoxetine and agomelatine can reverse the pathological phenotype of PRS rats by improving glutamate release, giving further evidence of the predictive validity of our model (Marrocco et al., 2012). Agomelatine was able to restore the levels of much SVPs in PRS rats, while fluoxetine had no effects on the expression of these proteins, suggesting two possible therapeutic pathways *via* the glutamatergic system where agomelatine acts through SVP-mediated glutamate release while fluoxetine-dependent glutamate restore seems not associated to SVP modulation. In the PRS model, depressive-like symptoms are on contrary associated with impairment in synaptic glutamatergic transmission, a profile also seen in the Flinders Sensitive Line (FSL, Overstreet and Wegener, 2013) rats, a

genetic model of depression (Nasca et al., 2013). mGlu2/3 receptor antagonists, such as MGS0039 and LY341495, (known to increase glutamate release (Nicoletti et al., 2011) had ATD-like effect in the rat FST and mouse tail-suspension test (Chaki et al., 2004), as well as in our model (Marrocco et al., 2012). Also, in the corticosterone (CORT)-treated rats, a treatment-resistant depression model, ketamine, which has been shown to increase glutamate transmission and synaptogenesis, possibly *via* inhibition of tonically active GABAergic interneurons (Duman and Aghajanian, 2012; Li et al., 2010) improved depressive-like behavior (Koike, Iijima and Chaki, 2013), acting on the presynaptic machinery (Muller et al., 2013). Accordingly, ketamine was shown to produce rapid (within hours) ATD responses in patients resistant to typical ATDs (reviewed by Krystal, Sanacora, Duman, 2013).

PRS rats are also characterized by an impairment in mGluR2/3 receptors expression (Zuena et al., 2008), than was also reversed by agomelatine (Morley-Fletcher et al., 2011).

Of note, N-acetylcarnithine, a drug which specifically enhances mGlu2 receptors in the hippocampus, is able to correct the depressive-like phenotype displayed by FSL rats (Nasca et al., 2013) and to reverse the deficit in glutamate release in these animals, suggesting a difference in the action of mGlu2 and mGlu3 receptors in the regulation of glutamatergic transmission in the hippocampus (Nasca et al., 2013). A distinct role for mGlu2 and mGlu3 metabotropic receptors has been indeed unraveled (Corti et al., 2007) and could account for the evidence that both mGlu2/3 receptor antagonists and agonists (or potentiators) exhibit antidepressant effects. In fact, glutamatergic abnormalities may differ, depending on brain regions and states of depression and may be either an increase or a decrease in synaptic glutamate release, so that mGlu2/3 receptor antagonists and agonists can be effective in different types of depression (Chaki et al., 2013). Interestingly, mGlu2/3 receptor antagonists and mGlu2/3 receptor agonists, assuming that both could have ATD potential, could be used for different types of patients. Because an mGlu2/3 receptor antagonist has been reported to have wake-promoting effect (Feinberg et al., 2005), they may be useful in patients with increased sleep, while mGlu2/3 receptor agonists which increase sleep (Feinberg et al., 2005) may be beneficial in patients with disturbed sleep patterns (Chaki et al., 2013).

PRS rats show alterations in circadian rhythm of motor activity and disturbances in sleep architecture (Mairesse et al., 2013), that can be considered as the key feature of their depressive-like phenotype. Agomelatine, which was able to improve hippocampal mGluR2/3 receptors of PRS rats (Morley-Fletcher et al., 2011) also corrected PRS-induced abnormalities in circadian rhythms (Mairesse et al., 2013).

2) Both male and female PRS rats display a depressive-like phenotype.

While the underlying neurobiological mechanisms involved in depression are likely multi-faceted, disturbed sleep is indeed reported by up to 90% of depressed subjects and might be involved, at least partially, in the onset and course of depression and in the response to treatment (reviewed by Palagini et al., 2013; Riemann, Berger and Voderholzer, 2001). Emerging evidence in both humans and rodents suggests that disturbances of the circadian system may contribute to mood disorders (Germain and Kupfer, 2008; McClung, 2007). Clock, one of the core circadian genes would be involved in reward drive, novelty-seeking, impulsivity, sleep patterns, and anxiety-/depression- like behaviors in mice (Le-Niculescu et al., 2009; Roybal et al., 2007), suggesting an important link between the circadian system and different aspects of emotionality. Furthermore, clinical literature indicates that polymorphisms in Clock and related genes are associated with mood disorders (Benedetti et al., 2003; Serretti et al., 2003).

Here, we were thus able to fully characterize the depressive-like phenotype in PRS male rats, giving evidence for altered circadian patterns of locomotor activity. PRS male rats display a phase advance in the onset of activity and inactivity and deficits in resynchronization after a challenge. These alterations were associated with an increase in hypothalamic corticotropin-releasing hormone (CRH) contents.

Here, for the first time, the behavioral phenotype was also analyzed in female rats; and we were able to demonstrate that female PRS rats, known to display a depressive-like phenotype in the forced swim test (Van Waes et al., 2006) and altered circadian patterns of corticosterone secretion (Koehl et al., 1999), also displayed alterations in circadian rhythms of locomotor activity. However, the profile obtained in PRS females showed differences in comparison to PRS males. This may be explained by the differential impact of PRS in males and females in term of anxiety, with male PRS rats showing an anxiety-like component in their depressive-like phenotype, while PRS females did not. Indeed, it has been shown that the expression of CRH receptors, in relation to anxiety-like behavior was differentially affected by prenatal stress in male and female rats (Brunton, Donadio and Russell, 2011) and CRH receptors antagonist exerts anxiolytic effect specifically in rats displaying innate anxious behavior (Keck et al., 2001).

These results are consistent with the observation that mood symptoms are often accompanied by significant differences in the diurnal rhythmicity of the HPA axis.

For instance, many depressed individuals exhibit dampened fluctuations in 24 h cortisol levels (Burke et al., 2005; Gold and Chrousos, 2002). Human postmortem studies have documented

gene expression alterations within brain regions that regulate the HPA axis, including increased CRH and arginine vasopressin (AVP) mRNA in the paraventricular nucleus of the hypothalamus (Bao, Meynen and Swaab, 2008), and altered ratios of GR and MR receptor mRNAs in the hippocampus of depressed patients (Lopez et al., 1998). CRH receptors antagonists were efficient in the treatment of depression (Holsboer, 1999).

3) PRS enhances addiction-like behavior for cocaine as well as for a natural reward such as chocolate.

Moving from the evidence that PRS exerts a specific effect in male and female rats, we wanted to extend the study of sex-dependent impact of PRS on vulnerability to drug addiction.

Here, we report that, independently of their prenatal history, females were more sensible than males to the locomotor activating effect of cocaine, consistently with several works, as already mentioned in the introduction (Lynch, Roth and Carroll, 2002). PRS enhanced locomotor activity in response to cocaine. Previously, it has been shown that a single injection of cocaine was sufficient to increase response in PRS rats (Kippin et al., 2008). Here, we have shown that a single injection was not sufficient to see the higher sensibility of PRS rats to the effects of cocaine on locomotion, consistently with the work of Henry et al. (1995), showing that enhanced behavioral sensitization to amphetamine in PRS rats was observed after several injections of the drug. Of note, we also report that PRS females, like males, display an increased locomotor response to cocaine after 6 days of chronic administration of the drug. There was, until now, only one study on the impact of cocaine in prenatally stressed male and female rats. This study also demonstrated that female PRS rats exhibit a strong behavioral response to cocaine after several injections, while no effect of PRS was observed in males (Thomas et al., 2009). Of note, the protocol used in this study, while being prenatal stress, differs somewhat as stress is applied later in gestation in comparison to our standard protocol.

Of note, response to cocaine-induced locomotor activity was predictive of the expression of preference toward cocaine CPP, as previously shown with amphetamine (Mathews, Morrissey and McCormick, 2010). Here, for the first time, we also demonstrated that PRS enhanced cocaine preference in PRS male rats in a CPP paradigm, giving further evidence of their increased vulnerability to drugs. Also, we were able to demonstrate the same profile of vulnerability in PRS females.

Moving from this evidence, we extended our study to chocolate as natural reward, to examine in the PRS well characterize model of enhanced vulnerability to drugs of abuse (Deminière et al., 1992; Koehl et al., 2000; Kippin et al., 2008; Morley-Fletcher et al., 2003; Reynaert et al., in preparation), how chocolate, could act like a drug.

Our data demonstrate that chocolate conditioned place preference induces specific changes in brain areas involved in hedonic feeding (hypothalamus) (Williams, 2014). Of note, genes modifications in the hypothalamus have been linked to the appetive power of salt (Liedtke et al., 2011). We have also demonstrate changes after chocolate CPP in the NAc, the key target structure of drugs of abuse (Nestler et al., 2005) as well in prefrontal cortex, also involved in addiction processes, in animals exhibiting an enhanced attraction for chocolate-paired chamber. The serotonergic system was greatly implicate in the behavioral response, and an increase in 5-HT_{2C} receptors expression was protective, a result which fits with the evidence of a positive effects of 5- HT_{2C} receptors agonists therapeutic potential in both obesity and drug abuse (reviewed by Higgings, Sellers and Fletcher, 2012). Moreover, we found that DA steady-state levels in the NAc were increased and decreased in male and female PRS rats, respectively. These data are in line with recent studies revealing the involvement of dopamine in what authors call “food addiction” (Baik, 2013), despite the lack of recognition of this evidence by the DSM, and the risk that this consideration will remain controversial for a long time (Berridge et al., 2010). This is quite surprising because the concept of food addiction was already evoqued by the group of Hoebel in 1988 (Hernandez and Hoebel, 1988). The question of chocolate as a drug was also asked (Bruinsma and Taren, 1999), and appears relevant as some people are describing themselves as “chocoholics” (Hetherington and MacDiarmid, 1993). Besides, the dopamine β-hydroxylase inhibitor, nepicastat, suppresses chocolate self-administration and reinstatement of chocolate seeking in rats (Zaru, 2013).

We were thus able, by using a well characterized model of addiction, to bring further elements in favor of the idea that chocolate could have an addictive power. From this study, it appears that females sensitiveness to drugs is stimulus-dependent, since PRS females appear in some way “protected” from sensibility to the reinforcing properties of chocolate while they are vulnerable to cocaine stimulus. Also in this part, sex played a key role in shaping rats profile.

4) Sex hormones exert a fundamental role in anxious/depressive disorders and addiction.

There are clear sex differences in the incidence and onset of stress-related and other psychiatric disorders, such as depression, anxiety and drug abuse in humans (Reviewed by Ter Horst et al., 2012) with high prevalence rates in women (Nolen-Hoeksema, 2001; Kessler

et al., 1995; Bekker and van Mens-Verhulst, 2007; Kessler, 2003; Lewinsohn et al., 1998; Steiner, Dunn and Born, 2003). However, women continue to be underrepresented in both epidemiological and clinical trials (Soldin and Mattison, 2009). Unfortunately, in preclinical studies, selection of male animals is also often the "default" choice and additional information related to sex in preclinical testing should be considered as the biological significance of sex hormones for mental health and disease is acknowledged (Raz and Miller, 2012; Zucker and Beery, 2010; Beery and Zucker, 2011). Sex hormones are indeed of great importance in determining anxious-depressive disorders, as observed both in humans and in animal models, and testosterone plays a key role in the onset of these disorders, as very recently reviewed by McHenry et al., 2014.

Here, we report that dihydrotestosterone (DHT) supplementation in control unstressed male rats, that confers to them a PRS-like profile is critical in inducing in them both an anxiety-/depressive and addictive-like profile.

Until know, most works on sex hormones in males have investigated the role of testosterone in modulating this kind of behaviors. Removal of an animal's testes—their primary source of endogenous androgens - through gonadectomy (GDX) - resulted in increased anxiety-like and decreased cognitive behavior (Frye and Seliga, 2001; Edinger and Frye, 2004).

In previous preclinical studies, testosterone (T) anxiolytic and antidepressant effect was demonstrated (Carrier and Kabbaj, 2012a; Frye and Walf, 2009). But, it has been shown that T beneficial effect was not mediated by T itself but *via* its conversion into estradiol (Carrier and Kabbaj, 2012b) or other T metabolites that bind estrogen receptors (Osborne, Edinger and Frye, 2009) while DHT did not induce any modification in anxiety/depression (Carrier and Kabbaj, 2012b; Svensson, 2012). Other studies report beneficial effect of DHT but DHT was injected and/or administered just before the behavioral tests (Edinger and Frye, 2005; Frye and Edinger, 2004). In an oldest work, a lowest ambulation and highest defaecation in the open field was found in DHT-treated animals, as a sign of anxiety-like behavior (Slob, Bogers and Van Stolk, 1981), consistently with our data.

In females, we have shown that ovariectomy in control unstressed rats induced anxiety-like behavior, consistently with other works (De Chaves et al., 2009). In PRS females, we have found a decrease in estradiol levels, which reached the levels obtained in ovariectomized control females. This suggests that a protective factor may counteract the impact of estradiol deficit in PRS females to protect them from anxiety, like progesterone.

Considering depressive-like behavior, ovariectomy also exerted critical effect, and we can assume that for this parameter, deficit in estradiol lies at the core of the phenotype obtained in

PRS females, even if we were not able, in this preliminary study, which needs further investigations, to demonstrate a beneficial effect of estradiol supplementation, although estradiol supplementation appears efficient in correcting behavioral despair in female rats (Walf and Frye, 2005). In PRS animals, hormonal manipulation failed in correcting well anxiety-/depressive-like profile, and others experimentations are needed to better characterize the impact of sex hormones in anxiety-/depressive-like profile of PRS animals. Also, human and animal studies indicate that drugs of abuse affect males and females differently, but the mechanism(s) underlying sex differences are still poorly known (Wissman et al., 2011).

In my PhD thesis, while DHT impact on addiction remains unknown, DHT supplementation in control unstressed males appears critical in inducing an enhanced response both to chocolate and cocaine, while finasteride decreased preference in males. An interesting hypothesis for translational studies is that DHT formation supports drug-seeking behavior in athletes taking testosterone esters, or that the use of 5α-reductase inhibitors in humans (e.g., for the treatment of benign prostatic hyperplasia or androgenetic alopecia) causes changes in hedonic sensitivity that are shaped by early life experiences. In females, estrogens have an established role in regulating the activity of the brain reward system (Galankin, Shekunova and Zvartau, 2010), and E_2 replacement or treatment with estrogen receptor ligands fully reverse changes in drug-seeking behavior and responses to psychostimulants caused by ovariectomy (Lynch, et al., 2001; Hu et al., 2004; Segarra et al., 2010; Jackson, Robinson and Becker, 2006). We found that E_2 replacement reversed the lowering effect of ovariectomy on hedonic sensitivity in unstressed female rats. In addition, E_2 supplementation in PRS female rats enhanced hedonic sensitivity to the same levels found in unstressed rats. Contrary to the effect of hormones found in chocolate CPP, ovariectomy increased cocaine-induced CPP in control rats to levels obtained in PRS females, consistently with another work (Bobzean et al., 2010). It appears that estrogens could exert a different impact in function of the intensity of stimuli, and that the anxiety or depression state plays a key role in modulating addictive-like behavior.

5) A self-medication hypothesis for the enhanced sensitiveness to drugs in PRS rats.

In the third chapter of the thesis, we wanted to underlie the comorbidity between anxiety, depression and addiction in the PRS model in rats. We were able to demonstrate an anxiolytic and antidepressive effect of chronic administration of cocaine in a disease-dependent manner. This effect of the drug was closely linked to the profile of preference for cocaine in rats. Our data provides thus evidence for a self-medication strategy in animals, with PRS rats of both sexes being more sensible to cocaine-induced CPP because of its antidepressive-like effect.

Interestingly, alcohol, while deleterious in control animals, was also shown to have beneficial effect in PRS female rats (Van Waes et al., 2011). The fact that sensitiveness to cocaine would be mediated by an improvement of depressive-like phenotype would explain why diazepam treatment was able to promote abstinence in rats from cocaine self-administration (Augier, Vouillac and Ahmed, 2012). Of note, rats treated repeatedly with cocaine, nicotine, or alcohol show significant anxiety-like responses following cessation of chronic drug administration (MerloPich et al., 1995; Rodriguez de Fonseca et al., 1997). Relapse would be due to these negative symptoms, and drug taking would be reinstated in an attempt to alleviate such symptoms.

If the self-medication hypothesis is well taken in account in humans (Khantzian, 1985; Markou, Kosten and Koob, 1998), it was very important to address this issue in the PRS model which recapitulates hallmarks of anxiety, depression and addiction. It appears from the present study that addiction in our model is a secondary symptom of anxiety/depression.

6) Alterations in glutamate transmission and mGlu receptors expression lies at the core of the behavioral abnormalities in the PRS model in rat.

We have shown in the first Chapter of the thesis that glutamatergic abnormalities lied at the core of the behavioral alterations and the depressive-like profile displayed by PRS rats, and that treatment with ATDs corrected PRS-induced disorders.

In Chapter 3, we have shown that cocaine exerted an action on glutamatergic system in the NAc. Our behavioral results showing that PRS male and female rats are more sensible to the rewarding effect of cocaine in CPP suggest that PRS would be more sensible to cocaine-induce glutamate release in the NAc. An increase in mGluR2/3 in the NAC of PRS rats would hence be a compensatory mechanism to respond to an enhanced cocaine-induced glutamate release (Kippin et al., 2008), as mGluR2/3 receptors negatively modulate glutamate transmission (Nicoletti et al., 2011). As we had shown, cocaine exerts anxiolytic and antidepressive action. The effect of cocaine on expression of mGlu receptors in the NAc would not only explain the response to the drug in CPP but also its beneficial effect in improving mood. Indeed, NAc is the key structure involved in reward. Impairments in reward circuitry have been found both in patients suffering from depression and patients with addictive disorders (Krishnan and Nestler, 2008). NAc would be therefore at the interface between cocaine rewarding and mood-enhancing effect (Russo and Nestler, 2013) and would have a fundamental role in depression (Sturm et al., 2003). A communication between hippocampus, which has been shown to be the key structure involved in the behavioral

abnormalities in our model (Marrocco et al., 2012; 2014), and the NAc could be involved (Belujon and Grace, 2011).

CONCLUSION

In conclusion, we have given further elements in favor of the high validity of the PRS model as a multifaceted model which allows the study of comorbid disorders with an integrated approach. We have given evidence for drug addiction as a self-medication strategy in treating anxious-/depressive-like profile.

Also, in our studies on anxiety, depression and addiction, we have demonstrated that the early-life stress interact with gender and sex hormones in determining such behaviors. PRS-like profile could be corrected by rebalancing hormonal status; and, we were also able to induce a PRS-like profile in control unstressed animals by modulating hormones. Together, these results suggest that an imbalance in sex hormones can lead to several disorders. This point raises a number of interesting questions in humans, e.g. how early life stress affects androgen or estrogen production and associated hedonic sensitivity and addictive behavior, or how estrogen or androgen treatment could have an impact in the response to drugs. Our results also pave the way to the development of new personalized therapeutical strategies that take into account the individual story. Finally, alterations of the glutamatergic system in the nucleus accumbens and ventral hippocampus could have a key role in shaping the comorbidity between stress-related disorders in the PRS model in rat.

PERSPECTIVES

To further investigate the action of cocaine as an antidepressant drug, we aimed at analyzing the expression or mGlu receptors in the ventral hippocampus. We will also analyze glutamate release in rats treated with cocaine, as we have seen that antidepressant drugs were able to correct abnormalities in glutamate release in PRS rats (Marrocco et al., 2014). It would be also interesting to assess animals response to cocaine CPP after a chronic treatment with antidepressants. Indeed, the hypothesis is that, when treated with antidepressant treatment, PRS rats would not self-medicate with drugs, so that their preference for cocaine would decrease. We also envisage the administration of mGlu receptors agonists, antagonists or allostatic modulators to further characterize the involvement of glutamate system in the

vulnerability to drug addiction and propose medications (Kalivas and Volkow, 2011) in the PRS model. We aim at examining the role of dopamine in the vulnerabily to cocaine in the PRS model (Koob and Nestler, 1997), in a context where PRS rats are shown to display changes in the expression and binding of dopamine receptors (Henry et al., 1995; Berger et al., 2002), increased excitation of DA neurons (Hausknecht, Haj-Dahmane and Shen, 2013) and to be hyper-responsive to the DA receptor agonist apomorphine (Marrocco et al., 2012) (Reviewed by Baier et al., 2012). We will aim at investigating the interaction between early-life stress and cocaine, in the connections between ventral hippocampus and NAc (Lipska et al., 1995), glutamate and dopamine pathways (Englom et al., 2008; Qi et al., 2011; Adrover, Shin and Alvarez, 2014). The PRS model appears a very valid multi-symptomatic model for dressing this issue (Deroche-Gamonet and Piazza, 2014).

As epigenetic factors are involved both in drug addiction and in depression (Renthal and Nestler, 2009), we will analyze the expression of some epigenetic regulators, that intervene with acetylation or methylation of DNA, such as HAT, HDACs, DNMT1, meCP2, in the nucleus of ventral hippocampus and NAc. We will also study the impact of the injection of HDAC inhibitors in rats response to cocaine self-administration (Romieu et al., 2008). As genome organizes long lasting sex differences in the brain *via* epigenetic differentiation (Kurian, Olesen and Auger, 2010), it would be interesting to address the question of epigenetics in sex hormones, in the PRS model, where hormonal alterations are seen (Pallarès et al., 2013, Reynaert et al., submitted). Another point would be the assessement of the glycosylation status of proteins that intervene in the gonadal steroids hormones production as glycosylation could modify the biological potency of gonadotrophins and modify estrogen and androgen production (Nussey and Whitehead, 2001).

Finally, we would also like to better understand the mechanisms of action of sex hormones and their role in anxiety/depression in females, by also studying progestogens that would be relevant in prenatal-stress processes (Frye et al., 2011).

The examination of the methodological issues concerning the aforementioned points is currently in progress.

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ABSTRACT

Stress is an important factor in the etiology of mood disorders and addictive behaviors. Prenatally restraint stressed (PRS) rats, i.e. the offspring of dams submitted to repeated episodes of stress during the last ten days of gestation display stress-related disorders such as anxiety- and depressive-like disorders but also increased vulnerability to drugs of abuse. An impairment of glutamate release in the ventral hippocampus lies at the core of the anxiety-like profile of PRS rats. Hence, we decided to investigate the effect of antidepressant treatment on glutamatergic system in our model. We found that chronic treatment with classical antidepressant drugs was able to enhance glutamate release and correct anxious-/depressive like profile of PRS male rats. Of note, a clear-cut sex effect has been shown in PRS-induced profile, with males being more anxious while male and female PRS rats display a similar depressive-like behavior. Here, for the first time, the alteration of circadian patterns, as a feature of depressive-like phenotype was analyzed both in male and female rats, and we have shown a gender-specific outcome of PRS on circadian systems modulating locomotor activity, the resynchronization to the new light-dark cycle, and hypothalamic CRH levels. Also concerning addiction, female profiles are less studied, so, we extended the impact of sex in our model to addiction. We have demonstrated that sex and in particular sex hormones played a key role in determining rats preference for drugs in a conditioned place preference paradigm, and that sensitiveness was expressed in a stimulus-dependent manner (chocolate as natural reward in comparison to cocaine). Finally, we found an enhanced preference for cocaine in females and in PRS rats of both sexes. This increase in preference for cocaine was linked to locomotor activating effect but also to the anxiolytic and antidepressant effect of the drug. This suggests that preference for a drug is enhanced when animals found a beneficial effect of this drug in correcting their mood disorders, reinforcing the hypothesis of self-medication in addictive-like disorders.

RESUME

Le stress est un facteur d'importance dans l'étiologie des troubles de l'humeur et des comportements addictifs. Des rats stressés prénatalement (PRS: prenatal restraint stress), i.e. la progéniture de femelles soumises à des épisodes répétés de stress les dix derniers jours de gestation, présentent des troubles liés au stress telles que des affections de type anxiété/dépression mais également une vulnérabilité aux drogues. Une diminution de la libération de glutamate dans l'hippocampe ventral s'avère au cœur du profil d'anxiété des rats PRS. Aussi, nous avons entrepris d'étudier dans notre modèle l'effet d'un traitement antidépresseur (ATD) sur le système glutamatergique. Nous avons montré qu'un traitement chronique avec des ATDs classiques était en mesure d'améliorer la libération de glutamate et de corriger le profil anxiodépressif des rats PRS mâles. Notons qu'un effet net du sexe a été mis en évidence dans le profil induit par le PRS, les mâles étant plus anxieux alors que les rats PRS mâles et femelles présentent un comportement de type dépressif similaire. Ici, pour la première fois, la modification des patterns circadiens, comme caractéristique de la dépression, a été analysée dans une même étude à la fois chez les rats mâles et femelles. Ainsi, nous avons montré un effet spécifique du sexe concernant l'impact du stress prénatal sur les systèmes circadiens, modulant l'activité locomotrice, la resynchronisation pour un nouveau cycle lumière-obscurité, et les niveaux de CRH hypothalamique. Nous avons ensuite étendu l'étude de l'influence du sexe dans notre modèle à la dépendance. Nous avons établi que le sexe, et en particulier les hormones sexuelles, jouent un rôle clé dans la détermination de la préférence des rats pour les drogues dans un paradigme de préférence de place conditionnée, et que la sensibilité était exprimée d'une manière stimulus-dépendante (chocolat comme récompense naturelle, comparativement à la cocaïne). Enfin, nous avons constaté que l'augmentation de la préférence pour la cocaïne était liée à l'effet d'activation de la locomotion, mais aussi à l'effet anxiolytique et antidépresseur de la drogue. Ceci suggère que la préférence pour une drogue est augmentée lorsque les animaux ressentent un effet bénéfique de cette drogue dans l'amélioration de leurs troubles de l'humeur, renforçant l'hypothèse de l'automédication dans les problèmes d'addiction.