N° d'ordre : ……

University of Lille 1: Sciences and Technologies

École Doctorale BIOLOGIE SANTÉ (ED n° 446)

PhD Thesis

European Label

Defended on March 8th 2016 by

Eleonora GATTA

LONG-TERM OUTCOME OF PERINATAL STRESS: TARGETING THE OXYTOCINERGIC SYSTEM IN THE EARLY PREVENTION OF STRESS-RELATED DISORDERS

Members of the Jury:

Prof. Christophe D'HULST	CNRS-University of Lille 1, France	***President of the Jury***
Prof. Anna PITTALUGA	University of Genoa, Italy	***Examiner and Internship Reporter***
Dr. Jean-Philippe PIN	Institut de Génomique Fonctionnelle, University of Montpellier 1 and 2, France	***Reporter***
Prof. John CRYAN	University College Cork, Ireland	***Reporter***
Prof. Peter FLOR	University of Regensburg, Germany	***Reporter***
Prof. Ferdinando NICOLETTI	CNRS-University of Lille 1, France Sapienza-University of Rome, Italy	***Supervisor***
Prof. Stefania MACCARI	CNRS-University of Lille 1, France	***Examiner***

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Prof. Stefania MACCARI	CNRS-University of Lille 1, France	***Examiner***

ABSTRACT

Both prenatal and postnatal factors (e.g., the transplacental transfer of molecules to the fetus and the extent and quality of matenal care, rexpectively) contribute to program the developmental trajectory of the offspring. This trajectory extends to the old age, which is the age at maximal risk for the clinical onset of chronic neurodegenerative disorders, such as Alzheimer's disease (AD).

A growing body of evidence has revealed the role of oxytocin as an antistress factor. In addition, during the *postpartum* period, maternal oxytocin plays a key role in mother-pup interactions that highly contribute to the development of the brain and shape the response of the hypothalamic-pituitary-adrenal axis to stress in the offspring. Using the model of prenatal stress in rats (PRS), we showed that postnatal administration of the oxytocin receptor agonist, carbetocin, to stressed mothers improved maternal behavior and prevented the pathological consequences of early-life stress across the entir lifespan of the offspring. These consequences include an anxious-/depressive-like phenotype as well as cognitive impairments and metabolic abnormalities in the aged offspring. We also demonstrated that chronic carbetocin treatment in adult rats was also able to correct the behavioral consequences of PRS and the underlying reduction in glutamate release in the ventral hippocampus, thus mimicking the action of the antidepressants fluoxetine and agomelatine.

Because we found a reduction in protein *O*-GlcNacylation in the hippocampus of aged PRS rats showing cognitive dysfunction, we also decided to examine whether a similar phenomenon was present in animals modeling AD. As a by-product of the main project we were able to demonstrate that AD mice carrying a triple mutation of amyloid precursor protein, presenilin-1 and tau show a selective reduction of protein *O*-GlcNacylation in the hippocampus. In particular, tau protein was hypo-*O*-GlcNacylated and hyperphosphorylated in the hippocampus of these mice. In conclusion, our data demonstrate that perinatal stress may represent a risk factor for psychiatric and neurodegenrative disorders and that carbetocin adminitration to either lactating mothers or the adult offspring may eliminate this risk. This raises the attractive possibility that mothers exposed to stress during gestation or in the early *postpartum* period or developing disorders that reduce maternal care such as *postpartum* depression should be treated with oxytocin receptor agonists to prevent the pathological consequences of a defective maternal care for the developing child.

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Thèse de Doctorat

Label Européen

Soutenue le 8 mars 2016 par

Eleonora GATTA

CONSÉQUENCES À LONG TERME DU STRESS PÉRINATAL : RÔLE DU SYSTÈME OCYTOCINERGIQUE DANS LA PRÉVENTION PRÉCOCE DES TROUBLES LIÉS AU STRESS

Membres du Jury:

Prof. Christophe D'HULST	CNRS-Université Lille 1, France	***Président du Jury***
Prof. Anna PITTALUGA	Université de Gênes, Italie	***Examinateur et Rapporteur de stage***
Dr. Jean-Philippe PIN	Institut de Génomique Fonctionnelle, Université Montpellier 1 et 2, France.	***Rapporteur***
Prof. John CRYAN	University College Cork, Irlande	***Rapporteur***
Prof. Peter FLOR	Université de Regensburg, Allemagne	***Rapporteur***
Prof. Ferdinando NICOLETTI	CNRS-University of Lille 1, France Sapienza-University of Rome, Italie	***Directeur de Thèse***
Prof. Stefania MACCARI	CNRS-Université Lille 1, France	***Examinateur***

RÉSUMÉ

Les facteurs pré- et postnataux (tels que le passage de molécules à travers la barrière placentaire ainsi que le degré et la qualité des soins maternels) contribuent tous deux à programmer le développement de la descendance. Cette programmation perdure jusqu'au vieillissement, période de risque maximal pour l'apparition clinique de maladies neurodégénératives chroniques telles que la maladie d'Alzheimer (MA).

De nombreuses études ont mis en évidence les propriétés antistress de l'ocytocine. En particulier, pendant la période périnatale, l'ocytocine maternelle intervient dans la mise en place des interactions mère-petit. Le comportement maternel joue un rôle important dans le développement du cerveau de la descendance et participe à la mise en place de la réponse de l'axe hypothalamo-hypophyso-surrénalien au stress. En utilisant le modèle de stress périnatal chez le rat (PRS), nous avons montré que l'administration postnatale à des mères stressées d'un agoniste du récepteur à l'ocytocine (la carbétocine) améliore le comportement maternel et empêche les conséquences néfastes du stress précoce tout au long de la vie de la descendance. Cela comprend le phénotype de type anxieux/dépressif, le déclin cognitif ainsi que les troubles métaboliques de la descendance âgée. Nous avons également démontré qu'un traitement chronique à la carbétocine durant la vie adulte corrige les altérations comportementales et la réduction de la libération de glutamate dans l'hippocampe ventral des rats PRS. Ainsi, la carbétocine a un effet similaire à celui des antidépresseurs conventionnels (fluoxétine, agomélatine). Au vu de la réduction en protéines *O*-GlcNacylées observée dans l'hippocampe des rats PRS âgés (présentant également un déclin cognitif), nous nous sommes alors demandés si un mécanisme similaire pouvait se retrouver dans des modèles animaux de la MA. Ainsi, nous avons pu démontrer que les souris portant une triple mutation sur la préséniline-1, la protéine précurseur de l'amyloïde, et la protéine tau montrent une réduction sélective de la *O*-GlcNacylation des protéines hippocampiques. En particulier, la protéine tau est hypo-*O*-GlcNAcylée et hyperphosphorylée dans l'hippocampe des ces souris.

En conclusion, l'ensemble de nos résultats montre que le stress périnatal peut représenter un facteur de risque pour le développement de troubles psychiatriques et des atteintes neurodégénératives. De manière intéressante, le traitement à la carbétocine chez des mères allaitantes, ou chez leur descendance adulte peut éliminer ce risque. Cela suggère que les mères exposées à un stress soit pendant la gestation, soit pendant la période postnatale, ou encore atteintes de troubles qui réduisent les soins maternels tels que la dépression *postpartum* pourraient être traitées par des agonistes des récepteurs à l'ocytocine afin de prévenir les conséquences néfastes induites par un soin maternel défectueux sur le développement de l'enfant.

Université de Lille 1, CNRS UMR8576

Unité de Glycobiologie Structurale et Fonctionnelle (Directeur : C. D'Hulst)

Equipe Glycobiologie des Maladies liées au Stress (Responsable : S. Maccari)

Laboratoire International Associé « Stress Prénatal et Maladies Neurodégénératives (Co-Directeurs : S. Maccari et F. Nicoletti)

To my mother,
Who early shaped my interest in the magic of the brain.

To my father,
Who encouraged me to follow my own path.

To Marc,
Thank you for walking by my side in the journey for PhD.

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"*Every stress leaves an indelible scar, and the organism pays for its survival after a stressful situation by becoming a little older.*"

Hans Selye

This last four-year path to PhD has been a considerable opportunity not only for building my conviction of pursuing scientific researches but also consisted in a continuous investigation of biological souls. Here, I would like to seize the opportunity to warmly thank my family, my old one and my new one (acquired in France) that unconditionally supported and encouraged me all along this journey.

I wish to express my deep gratitude to Prof. Christophe D'Hulst, Head of the Structural and Functional Glycobiology Unit, for providing over the years an excellent scientific environment I could share with my colleagues as well as for his availability to be member of my PhD Jury.
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LIST OF ABBREVIATIONS

2-OG	2-oxoglutarate
5-HT	5-hydroxytryptamine, serotonin
11β-HSD1	11β-hydroxysteroid-dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid-dehydrogenase type 2
AAT	aspartate aminotransferase
Aβ	amyloid-β plaques
ABN	arched-back nursing
AC	adrenal cortex
ACTH	adrenocorticotropic hormone, corticotropin
AD	Alzheimer's disease
Ad	adrenalin
ADHD	attention deficit/hyperactivity disorder
AM	adrenal medulla
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	activator protein-1
APP	amyloid precursor protein
Aralar	aspartate-glutamate carrier
Asp	aspartate
AVP	(arginin)-vasopressin
BDNF	brain-derived neurotrophic factor
BNST	bed nucleus of stria terminalis
Ca^{2+}	calcium
Ca^{2+}/CaM	Ca^{2+}-calmodulin complex
CA	cormu ammonis
CAMK	Ca^{2+}/Calmodulin-dependent protein kinase
CB1	cannabinoid receptor 1
CBG	corticosteroid binding protein, transcortin
CeA	central nucleus of the amygdala
CICR	Ca^{2+} -induced Ca^{2+} release
CNS	central nervous system
COX	cyclooxygenase 2
CRH	corticotropin-releasing hormone
CRHR1	type-1 CHR receptors

DAG	diacylglycerol
DG	dentate gyrus
DOHaD	developmental origins of health and disease
DRN	dorsal raphe nucleus
EAAT	excitatory amino acid transporter
EC	entorhinal cortex
ECB	endocannabinoids
FKBP12	FK-506 binding protein-type 12
FTD	frontotemporal dementia
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
GAS	general adaptation syndrome
GAT	GABA transporter
GBS	GR-binding sites
GC	glucocorticoids
GDH	glutamate dehydrogenase
gln	glutamine
GLUT	glucose transporter
glut	glutamate
GRs	glucocorticoid receptors
GREs	glucocorticoids regulatory elements
GSK3β	Glycogen Synthase Kinase 3β
HBP	hexosamine biosynthetic pathway
HPTMs	posttranslational modifications of histones
Hsp	heat-shock protein
IGF-1	insulin-like growth factor-1
iGluRs	ionotropic glutamate receptors
il	infralimbic
InsP3	inositol trisphosphate
KO	knockout
LC	locus coeruleus
LG	licking and grooming
LTD	long-term depression
LTP	long-term potentiation
LPP	lateral perforant path

HPA	hypothalamic-pituitary-adrenal
mGluR	metabotrobic glutamate receptors
MC2	type-2 melanocortin receptors
MeA	medial amygdaloid nucleus
MF	mossy fibers
MPP	medial perforant path
MSH	melanocyte-stimulating hormone
mPFC	medial prefrontal cortex
mpPVN	medialparvocellular paraventricular nucleus
MRs	mineralocorticoid receptors
NAd	noradrenaline
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NARI	monoaminooxydase inhibitors, noradrenaline reuptake inhibitors
NASSA	noradrenergic specifically serotonergic antidepressants
NF-κB	nuclear factor-κB
NFT	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NOS	NOsynthase
NPCs	neural progenitor cells
NR3C1	nuclear receptor subfamily 3, group C, member 1)
NTS	*nucleus tractus solitarii*
OGA	*O*-GlcNAcase
OGT	*O*-GlcNAc transferase
O-GlcNAc	*O*-N-acetylglucosamine
OTR	oxytocin receptor
PD	Parkinson's disease
PFC	prefrontal cortex
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C
Pl	prelimbic
PND	postnatal day
POA	preoptic area
POMC	pro-opiomelanocortin
PRS	perinatally stressed
PTSD	post-traumatic stress disorder

PVN	paraventricular nucleus
ROK	Rho kinase
RRP	readily releasable pool
RyR	ryanodine receptor
Sb	subiculum
SC	Schaffer collateral
SCN	suprachiasmatic nucleus
SHRP	stress hyporesponsiveness period
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNRI	serotonin/noradrenaline reuptake inhibitors
SON	supraoptic nuclus
SSA	succinic semialdehyde
SSRI	selective serotonin reuptake inhibitor
sPVN	subparaventricular nucleus zone
TA	temporoammonic pathway
TCA	tricarboxylic acid cycle
UDP-GlcNAc	uridine diphosphate N-acetylglucosamine
USVs	ultrasonic vocalizations
VAMP	vesicle-associated membrane proteins
vlDMH	ventrolateral region of the dorsomedial hypothalamic nucleus
VFT	Venus fly trap
vGAT	vesicular GABA transporter
VGCC	voltage-gated Ca^{2+} channels
VGLUT	vesicular glutamate transporters
vSUB	ventral subiculum
Xc^-	cystine-glutamate antiport

GENERAL SUMMARY

Several epidemiological studies have highlighted the consequences of maternal distress on the behavioral, neurological and endocrine outcome of the offspring. Both prenatal and postnatal factors contribute to program the developmental trajectory of the offspring. This trajectory extends to the old age, which is the age at maximal risk for the clinical onset of chronic neurodegenerative disorders, such as Alzheimer's disease. Indeed, vulnerability to develop chronic diseases may be programmed early in life, and, in particular, during the *peripartum* period, which is critical for shaping the lifelong health of an individual (Barker, 1995). This led to the elaboration of the theory of the developmental origins of health and disease (DOHaD).

Early exposure to environmental stressors may thus have a long-lasting effect on the developing organism, thereby programming or "imprinting" persistent changes in the offspring. Preclinical and clinical studies have shown that the brain is particularly sensitive to stress during infancy. Interestingly, the same occurs during aging. Thus, infancy and aging are considered as "critical periods" for the brain response to stress (Lupien et al., 2009). Early stressful events may produce either adaptive or maladaptive consequences in the offspring (Gluckman and Hanson, 2004), which are initially aimed at supporting survival, but then predispose the individual to later life disease, particularly if the environment diverges from that predicted by early life conditions. If so, early life events may cause the late development of several neurological, metabolic and neuroendocrine abnormalities (Seckl, 1998; Lupien et al., 2009) that can persist all life long.

During the last decades, different animal models have been developed in which stress is applied to pregnant dams or early in life, and the outcome is examined across the entire life span of the offspring. I used the model of perinatal stress in rats originally described by Prof. Stefania Maccari, in which pregnant dams are exposed to multiple episodes of restraint stress. The offspring of stressed dams (here, called perinatally stressed rat or "PRS" rats) develop long-lasting biochemical and behavioral changes that likely reflect the induction of a pathological programming caused by early-life stress. Adult PRS rats develop an anxious-/depressive-like phenotype characterized by a prolonged hypothalamic-pituitary-adrenal (HPA) stress response (Maccari *et al.,* 1995, Darnaudéry and Maccari, 2008), high plasma glucose levels associated with low body weight (Vallée *et al.,* 1996), and also a reduction of neurogenesis in the hippocampal dentate gyrus (Morley-Fletcher *et al.,* 2011). The ventral part of the hippocampus,

which encodes memories related to stress and emotions (Fanselow and Dong, 2010), is particularly vulnerable to PRS. Remarkably, most of the alterations induced by PRS are reversed by chronic antidepressant treatment (Morley-Fletcher et al., 2011; Mairesse *et al.,* 2013; Marrocco *et al.,* 2014), as well as by early adoption (Maccari *et al.,* 1995). In addition, adrenalectomy in the mother, which abolishes glucocorticoid secretion, prevents the long-term consequences of perinatal stress (Barbazanges *et al.,* 1996). Besides the anxious-/depressive-like phenotype in the adult life, others symptoms appear during aging in PRS rats. Aged PRS rats exhibit cognitive and metabolic dysfunctions, as reflected by impairment in spatial learning (Vallée *et al.,* 1999) and abnormalities in glucose metabolism (Lesage *et al.*, 2004).

The behavioral and neuroendocrine phenotype of the offspring is the result of a complex interaction between prenatal, genetic and postnatal factors. While glucocorticoids play an important role in fetal development, other endocrine systems contribute to the developmental trajectory of the offspring in the early postnatal life. During the *peripartum* period maternal oxytocin not only stimulates lactation, but also enhances mother-pup interactions (Neumann and Landgraf, 2012), thus influencing brain development and shaping the response of the hypothalamic-pituitary-adrenal (HPA) axis to stress in the offspring (Windle et al., 1997; 2004). However, the involvement of oxytocin in the early phases of the pathological programming induced by gestational stress, and in the long-lasting expression of the PRS phenotype is lagely unknown. Knowing that perinatal stress causes alterations in mother-pup interactions (Patin et al., 2002), we have predicted that treatment of stressed mothers with drugs that activate oxytocin receptors could prevent the development of the pathological phenotype triggered by perinatal stress, and, therefore, reduces the risk for CNS disorders late in life. Indeed, a reduced maternal care soon after birth highly contributes to the development of the abnormal HPA response to stress and other "pathological" hallmarks induced by prenatal stress in the offspring (reviewed by Weaver, 2009).

The aim of my PhD thesis was to establish whether alterations induced by early-life stress could be extended to the old age of the offspring, and, more important, whether the pathological phenotype of PRS rats could be prevented or reversed by pharmacological activation of oxytocin receptors in lactating mothers or in the adult offspring.

In the first part of the thesis, I show that postnatal administration of the oxytocin receptor agonist, carbetocin, to stressed mothers improves maternal behavior and prevents the development of the pathologic phenotype in PRS rats. Maternal carbetocin treatment could also prevent cognitive dysfunction and metabolic abnormalities exhibited by PRS rats during

ageing. This finding is particularly interesting because of the tight relationship between a defective glucose metabolism and the development of Alzheimer's disease and other types of dementia. One potential implication of my study is that treatments that improve maternal care, including oxytocin receptor agonists, may reduce the risk for Alzheimer's disease in the old age. To strengthen the relationship between abnormalities in glucose metabolism and age-related cognitive disorders I was also involved in a side project demonstrating that *O*-GlcNAcylation of brain proteins in general and tau protein in particular is reduced in the hippocampus of transgenic mice modeling Alzheimer's disease.

In the second part, it is shown that adult treatment with carbetocin reverses all behavioral and neurochemical alterations induced by PRS by particularly targeting the glutamatergic synapse in the ventral hippocampus. The effect of carbetocin was similar to that produced by the conventional antidepressants, fluoxetine and agomelatine.

Taken together, data presented and discussed in this PhD thesis (i) strengthen the view that changes in either prenatal and/or postnatal environment may cause permanent modifications in the developmental trajectory of the offspring, thus shaping the vulnerability to CNS disorders later in life; (ii) show that a reduced maternal care is critical for the pathological programming induced by perinatal stress; and, (iii) suggest that pharmacological activation of oxytocin receptors in lactating mothers or in the adult offspring is a valid strategy to prevent or reverse the pathological phenotype caused by perinatal stress, including abnormalities in glucose metabolism that predispose to the onset of cognition disorders during ageing.

RÉSUMÉ GÉNÉRAL

De nombreuses études épidémiologiques montrent que l'exposition de la mère à un environnement aversif durant la période périnatale entraîne des altérations comportementales, neurologiques et endocrines chez la progéniture. Les périodes pré- et post-natales contribuent à programmer le développement de la descendance. Cette programmation perdure jusqu'au vieillissement, période de risque maximal pour l'apparition clinique de maladies neurodégénératives chroniques telles que la maladie d'Alzheimer. En effet, la vulnérabilité à développer des maladies chroniques peut être programmée de manière précoce, et, en particulier, au cours de la période périnatale. Cette programmation est essentielle pour déterminer à long terme l'état de santé d'un individu (Barker, 1995). Cette hypothèse a conduit à l'élaboration de la théorie des origines développementales de la santé (*Developmental Origin of Health and Disease,* DOHaD).

L'exposition précoce aux stress environnementaux peut donc avoir un effet à long terme sur l'organisme en développement, conduisant ainsi à la programmation ou "l'empreinte" de changements persistants chez la descendance. Les études précliniques et cliniques ont montré que le cerveau est particulièrement sensible au stress pendant l'enfance, ainsi qu'au cours du vieillissement. Ainsi, ces deux périodes sont considérées comme des «périodes critiques» pour la mise en place de la réponse du cerveau au stress (Lupien et al., 2009). L'exposition précoce au stress peut induire le développement chez la descendance d'un phénotype adapté ou inadapté (Gluckman et Hanson, 2004). D'un point de vue évolutif, ce phénotype vise initialement à promouvoir la survie de l'individu, mais prédispose par la suite au développement de pathologies au cours de la vie, en particulier lorsque les conditions environnementales divergent de celles prédites par la vie précoce. Ainsi, l'exposition au stress au cours de de la vie précoce peut être responsables du développement de troubles neurologiques, métaboliques et neuro-endocrines (Seckl, 1998; Lupien et al., 2009) pouvant persister tout au long la vie.

Au cours de ces 30 dernières années, différents modèles animaux ont été développés. Certains de ces modèles consistent à exposer des femelles gestantes, ou leur jeune descendance, à un stress chronique. Au cours de cette thèse, j'ai utilisé le modèle de stress périnatal chez le rat, modèle développé par le Pr. Stefania Maccari, dans lequel les femelles gestantes sont exposées à de multiples épisodes de stress de contention. La descendance des femelles stressées (appelée

ici stressée périnatalement ou rats "PRS") développe des altérations biochimiques et comportementales durables qui reflètent vraisemblablement l'induction d'une programmation pathologique causée par le stress précoce. Les rats PRS adultes développent un phénotype de type anxieux/dépressif caractérisé par un réponse prolongée de l'axe hypothalamo-hypophyso-surrénalien (HHS) suite à un stress (Maccari et al., 1995, Darnaudéry et Maccari, 2008), des niveaux de glucose plasmatique élevés associés à un faible poids corporel (Vallée et al., 1996), et également une réduction de la neurogenèse dans le gyrus denté de l'hippocampe (Morley-Fletcher et al., 2011). Ainsi, la partie ventrale de l'hippocampe, qui est responsable de la mémoire liée au stress et aux émotions (Fanselow et Dong, 2010), est particulièrement vulnérable au PRS. De façon intéressante, la plupart des altérations induites par le PRS sont corrigées par un traitement chronique aux antidépresseurs (Morley-Fletcher et al., 2011; Mairesse et al., 2013; Marrocco et al., 2014), ainsi que par une adoption précoce par des mères non stressées (Maccari et al ., 1995). De plus, la surrénalectomie chez la mère, qui abolit la sécrétion de glucocorticoïdes, prévient le développement des conséquences néfastes induites par le stress périnatal (Barbazanges et al., 1996). En plus du phénotype de type anxieux/dépressif observé chez les rats PRS adultes, d'autres symptômes apparaissent plus particulièrement au cours du vieillissement. En effet, les rats PRS âgés présentent des dysfonctionnements cognitifs et métaboliques, e.g., les troubles de l'apprentissage spatial (Vallée et al., 1999) et les anomalies du métabolisme du glucose (Lesage et al., 2004).

Le phénotype comportemental et neuroendocrinien de la descendance est le résultat d'une interaction complexe entre des facteurs prénataux, génétiques et postnataux. Bien que les glucocorticoïdes jouent un rôle important dans le développement du fœtus, d'autres systèmes endocriniens contribuent au développement de la progéniture dans la vie postnatale précoce. Au cours de la période périnatale, l'ocytocine maternelle stimule la lactation, mais intervient également dans l'interaction mère-petit (Neumann et Landgraf, 2012), influençant ainsi le développement du cerveau et participant alors à la mise en place de la réponse au stress de l'axe HHS chez la descendance (Windle et al., 1997; 2004). Cependant, l'implication de l'ocytocine dans les premières phases de la programmation pathologique induite par le stress gestationnel, et dans l'expression à long terme du phénotype PRS n'est que très peu connue. Etant donné que le stress périnatal provoque des altérations dans l'interaction mère-petit (Patin et al., 2002), nous avons émis l'hypothèse que le traitement des mères stressées par des molécules activant les récepteurs de l'ocytocine pourrait empêcher le développement du phénotype pathologique induit par le stress périnatal, et, par conséquent, réduire le risque de

développer de troubles du système nerveux central (SNC) au cours de la vie. En effet, la réduction de soins maternels peu après la naissance contribue fortement au développement de la réponse anormale au stress de l'axe HHS ainsi qu'au phénotype anxieux/dépressif et au vieillissement anticipé de la descendance stressée périnatalement (Weaver, 2009).

Le but de ma thèse de doctorat a été d'établir si les altérations induites par le stress précoce pourraient perdurer au cours du vieillissement, et, plus particulièrement, si le phénotype pathologique de rats PRS pourrait être enrayé ou corrigé par l'activation pharmacologique des récepteurs à l'ocytocine chez les mères allaitantes ou chez la descendance adulte.

Dans la première partie de cette thèse, il est montré que l'administration postnatale d'un agoniste des récepteurs à l'ocytocine, la carbétocine, améliore le comportement maternel des femelles stressées et prévient le développement du phénotype pathologique des rats PRS. Le traitement maternel à la carbétocine prévient également le dysfonctionnement cognitif ainsi que les anomalies métaboliques des rats PRS vieillissants. L'ensemble de ces résultats est particulièrement intéressant en raison de la relation étroite existant entre un défaut du métabolisme du glucose et le développement de la maladie d'Alzheimer et d'autres types de démence. Ainsi, les traitements qui améliorent les soins maternels, notamment les agonistes des récepteurs à l'ocytocine, semblent être de bons candidats pour réduire le risque de développer la maladie d'Alzheimer au cours du vieillissement. Afin de mettre en évidence la relation existant entre les anomalies du métabolisme du glucose et les troubles cognitifs liés à l'âge, j'ai également mis en évidence que la *O*-GlcNAcylation des protéines du cerveau et de la protéine tau en particulier, est réduite dans l'hippocampe de souris transgéniques utilisées comme modèle animal pour l'étude de la maladie d'Alzheimer.

Dans la deuxième partie, il est montré que le traitement à la carbétocine, pendant la vie adulte, renverse toutes les modifications comportementales et neurochimiques induites par le PRS en ciblant particulièrement la synapse glutamatergique de l'hippocampe ventral. L'effet de la carbétocine est similaire à celui des antidépresseurs classiques (fluoxétine, agomélatine).

L'ensemble des données présentées et discutées dans cette thèse (i) renforce l'idée que des changements survenant dans l'environnement pré- et/ou postnatal peuvent causer des modifications permanentes au cours du développement de la progéniture, entrainant ainsi la mise en place de la vulnérabilité aux troubles du système nerveux central au cours de la vie; (ii) montre que la diminution des soins maternels joue un rôle clé dans programmation pathologique induite par le stress périnatal; et, (iii) suggère que l'activation pharmacologique des récepteurs à l'ocytocine chez les mères allaitantes ou chez la descendance adulte est une

stratégie intéressante pour prévenir ou inverser le phénotype pathologique causé par le stress périnatal, y compris les anomalies du métabolisme du glucose qui prédisposent à l'apparition de troubles de la cognition au cours vieillissement.

INTRODUCTION

1. Stress: where does it start?

Life of all living organisms continuously goes on in an interacting environment. Ability to survive depends on the ability to cope with a myriad of stimuli coming from the external environment. Hence, the organism should interpret and integrate this incoming information to maintain its equilibrium and adapt to environmental disruptors. Hippocrates (c. 460 – c. 370 BC) highlighted that disturbances of the steady state in the four humors of an organism (i.e. blood, phlegm, yellow and black bile) could be the cause for disorders. Nevertheless, a living organism should keep a "steady-state" independently of what happens in the surroundings. In 1878, Claude Bernard developed the idea that the inner organism maintains a balance by introducing the concept of "milieu intérieur". This new physiological concept defined how the good functioning of a working system depends on a fine balance of all its components but also on the interaction among them in spite of any external stimulation.

In 1932, Walter Cannon introduced the notion of "homeostasis". He wrote that "the constant conditions which are maintained in the body might be termed *equilibria*." Hence, homeostasis, from the Greek ομοιος, "similar" and στασις, "remain still", is the property of an organism to maintain and regulate its inner variables in order to keep internal conditions stable and relatively constant over time. Stimuli that the organism perceives as threatening against the reached equilibrium are nowadays considered as "stressors" (Lazarus and Folkman, 1984). In response to threat, an adequate physiological response is essential to survive.

In 1935, Hans Selye was the pioneer in the definition of "stress" as the non-specific response of the organism to a stimulus. In reaction to new information perceived, the organism activates biological systems in order to give a behavioral reaction to stress. The response to stress can be subdivided into three phases of response to stress, defining the "General Adaptation Syndrome" (GAS) (Selye, 1950; 1976). First, the organism perceives the stimulus as a new condition. This will trigger the "alarm reaction" phase, during which the organism needs to mobilize energetic resources. During this alarm state, endocrine systems must rapidly cope with the stressor. Catecholamines such as epinephrine and norepinephrine are produced by the sympathetic system, with resulting enhanced muscular tone, blood pressure and mobilization of energy store enabling an adapted response to stress, i.e. the "fight-or-flight" response described by Cannon and De La Paz (1911). Second, the phase reaction to stress called resistance. It consists in a

slower response during which the organism attempts to resist to stress (by the activation of neuroendocrine systems) and restores its equilibrium, homoeostasis. Finally, if stress persists, there is a third phase of exhaustion that could lead to a pathological state. The organism is exhausted by the first aggression and every new negative stimulus becomes a challenge. While responses to acute exposure to stress may be beneficial for the defense of the organism, repeated exposure to stressful events might be detrimental. The response to stress is very energy-dependent and needs physiological activation of endocrine systems (see below) with appropriate feedback regulations to be efficient.

In the end, the notion of stress revolves around three main components (reviewed by Van Reeth et al., 2000; Fig.1):

- Stimulus (positive or negative);
- Cognitive response to the stimulus (strongly related to life experience and predictive abilities);
- Physiological response of the individual.

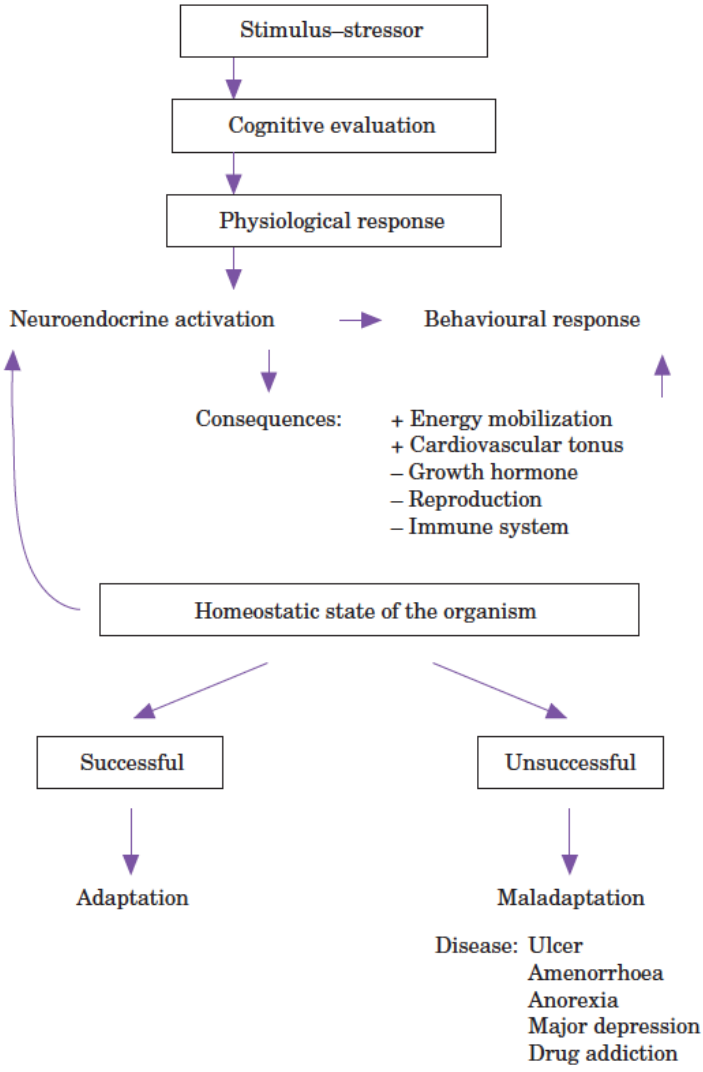

Figure 1: Schematic representation of a multidimensional concept of stress build around three important concepts: the stimulus-stressor, its cognitive evaluation by the organism and resulting physiological response(s).
Activation of various biological systems (including neuroendocrine and behavioural components) leads to resistance of the organism, resulting in activated biological systems returning to normal. If the source(s) of stress are prolonged and/or uncontrollable, feedback mechanisms fail in restoring the equilibrium, the stress response becomes inadequate and may ultimately result in various pathological states, including sleep and mood disorders (adapted from Van Reeth et al., 2000).

The concept that the organism has to regain equilibrium after exposure to stress was reconsidered in the late 80's. Opposite to the notion of homeostasis, where the equilibrium is considered to be constant and true to form, Sterling and Eyer (1988) introduced a new concept, defined as "allostasis", in which the response to stress causes changes in the current state to reach a different equilibrium. This suggests that if an appropriate response to stress is needed, it does not necessarily imply a return to the "status quo ante", but instead requires an adaptive process, thus "remaining stable by being variable" (Sterling and Eyer, 1988). Allostasis becomes a crucial process for the organism to adapt to predictable and unpredictable events. Nevertheless, persistency of adverse events may lead to discrepancies in the organism ability to cope with stress and to an inadequate physiological response that is very energetically costly, i.e. an "allostatic load" followed by an "overload" (McEwen, 1998). This lays the groundwork for appearance of stress-related disorders, particularly involving the cardiovascular and immune systems (McEwen and Stellar, 1993).

The brain plays a crucial role to orchestrate and adequately adapt the response to the challenge of the stressor (for review McEwen and Gianaros, 2011; McEwen, Gray and Nasca, 2015). In response to environmental changes, the brain readjusts and actively modifies its morphology, a phenomenon called "neuronal plasticity" (Zilles et al., 1992). This ability is particularly relevant to functionally adapt to the environment and is required for activation of specific neuroendocrine systems. Activation of specific systems to give an adequate stress response represents an evolutionarily conserved ability of an organism to deal with circumstances that require vigilance, arousal, and/or action (Nesse, Bhatnagar and Young, 2007).

The majority of stress responses need previous interpretation of the potential threat coming from the environment. A given stimulus is thus compared to prior experiences or to an innate program. This "psychogenic response" is necessary for the organism to gather the energy to either avoid or fight the challenge in order to maximize the potential for survival (Herman et al., 2003). Anticipatory responses involve cortico-limbic forebrain structures (hippocampus, medial prefrontal cortex (mPFC), and amygdala) (Ulrich-Lai and Herman, 2009) processing sensory information and regulating emotion, reward, and mood. Thus, this anticipation is the result of either conditioning (related to memory formation) or innate species-specific predisposition (e.g. identification of a predator and fear response). The mnemonic aspects of anticipatory stressors are important determinants of the response to stressful stimuli. Nevertheless, there are subjective differences in the perception of a stressful event and the adequate response to stress lies in a complex interplay between physiological, psychological,

and behavioral processes. Responses to threats are highly influenced by genetics and lifelong history of stress-exposure, and are a very important component of stress-related diseases, such as anxiety/depression, posttraumatic stress disorder (as well as many systemic diseases) and age-related disorders. As discussed above, the response to stress is energetically costly and cannot be over-engaged without deleterious consequences (McEwen, 1998). Finally, the brain generates memory-dependent inhibitory and excitatory pathways to control responses to stress and prepare for future demand. Stress response requires a dynamic circuit that involves activation of specific central and peripheral structures during stress exposure.

1.1. Integrative circuitry of the stress response and neuroendocrine mediators: focus on glucocorticoids and oxytocin

Physiological response to stress requires the activation of neuroendocrine systems. First evidence that sensorial perception of environmental stimuli was linked to specific inputs in the brain was provided by Geoffrey Harris (1972), who is today considered the father of Neuroendocrinology. He established the existence of a communication between the brain and the body mediated by the neuroendocrine system. In particular, Harris highlighted the existence of a central control of the pituitary-adrenal activity *via* the hypothalamus, suggesting that the hypothalamus communicates with the anterior pituitary through the systemic circulation, i.e. the portal vascular system (Harris, 1970). In the second half of the twentieth century, researches were particularly flourished in the field of Neuroendocrinology and that was the time for the discovery of hypothalamic releasing factors (Schally, Arimura and Kastin, 1973; Guillemin, 1978, Vale et al., 1981).

The main actor of the stress response is the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 2). Corticotropin-releasing hormone (CRH) and vasopressin (AVP) are the two upstream hypothalamic hormones essential in activating behavioral and endocrine response to stress. Successful reaction to stress needs appropriate regulation of the neuroendocrine stress response that goes beyond the strict definition of the HPA axis and has both central and peripheral components. The endocrine response to stress follows a specific time-course and is restricted over time in order for the individual to adapt to new conditions. This hormonal vertebrate response to stress consists in two main phases of hormonal cascade with major physiological consequences (Sapolsky, Romero and Munck, 2000). A critical feature of the activation of the reflexive stress cascade is to mobilize energy for immediate use while simultaneously inhibiting body functions that are nonessential for survival in that moment.

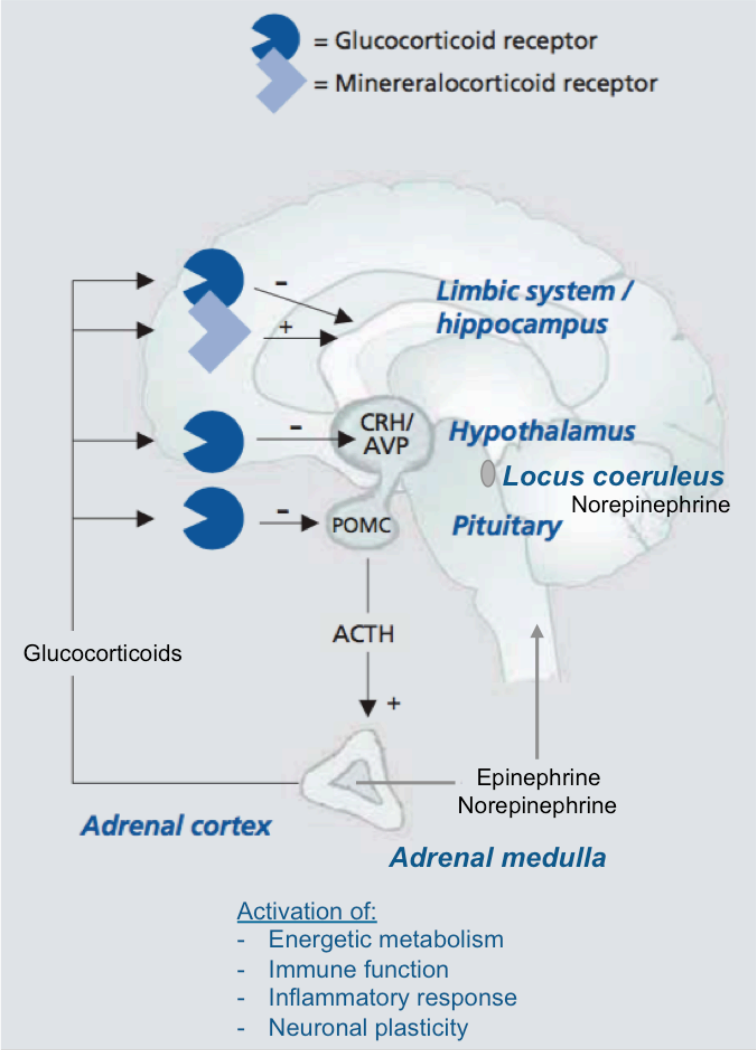

Figure 2: Regulation of the Hypothalamic-Pituitary-Adrenal axis.
CRH, corticotropin-releasing hormone; AVP, arginin-vasopressin; POMC, pro-opiomelanocortin; ACTH, adrenocorticotropic hormone (adapted from Ising and Holsboer, 2006).

1.1.1. A prompt response to stress: activation of the sympathomedullary system

In the few seconds following stressor exposure, the autonomic sympathomedullary system enhances secretion of catecholamines: epinephrine and norepinephrine [or noradrenaline] (Womble et al., 1980). Catecholamines mediate the short-term response to stress allowing the organism to rapidly cope with the threat perceived.

a) Direct Pathways and initiation of the stress response

Epinephrine released from the adrenal medulla in the portal circulation induces vasoconstriction with an ensuing increase in blood pressure by activating α_1-adrenergic receptors (Hoffman, 2001). Also, epinephrine stimulates norepinephrine release by β-adrenergic receptors on sympathetic afferents. Central noradrenergic nuclei (e.g., neurons of the locus coeruleus [LC]) are also activated in response to stress. This contributes to the activation of the cardiovascular system and the HPA axis, increases arousal, and activates

specific cognitive domains (such as attention and vigilance) that are essential for the response to stress. Activation of basolateral amygdala, hippocampus and mPFC by norepinephrine is essential for consolidation of emotional memories (Roozendaal, McEwen and Chattarji, 2009). As a consequence of catecholamines release, there is a cascade of physiological events including increased respiration and heart rate, dilation of skeletal muscle blood vessels, enhanced glucose production from the liver, and vasoconstriction of digestive and reproductive organ blood vessels (Thiel and Dretsch, 2011, Table 1). Activation of sympathomedullary system is necessary for a rapid mobilization of energetic resources, enhancing blood flow with an ensuing increase in oxygen and glucose provision to skeletal muscles and brain tissues (McCarty, 2000). Hence, the body is prepared to action: "fight or flight" in response to the threat perceived.

Brainstem noradrenergic projections reach the paraventricular nucleus (PVN) of the hypothalamus to activate corticotropin-releasing hormone (CRH) release (Petrov, Krukoff and Jhamandas, 1993; Whitnall, Kiss and Aguilera, 1993) with an ensuing activation of the HPA axis and physiological adaptation to stress (Table 1). Of note, the noradrenergic system has been shown to exert a direct control of the HPA axis activity (Maccari et al., 1992). CRH, a 41 amino acids peptide, is the main hypothalamic regulator of the HPA axis. Since CRH was first discovered (Vale et al., 1981), different CRH receptor subtypes and a growing number of receptor ligands have been identified. CRH and CRH receptors are widespread in extrahypothalamic brain regions including limbic regions, the basal forebrain, and the LC-noradrinergic sympathetic system in the brainstem and spinal cord (Charmandari, Tsigos and Chrousos, 2005). CRH is the prototypic ligand of type-1 CHR receptors (CRHR1), which are coupled to G_s proteins (Bale and Vale, 2004).

There is a reciprocal connection between PVN and noradrenergic neurons in the LC, with CRH and norepinephrine stimulating each other through CRHR1 and α_1-adrenergic receptors, respectively (Valentino, Foote and Aston-Jones, 1983; Kiss and Aguilera, 1992). Autoregulatory feedback loops are present between the PVN CRH neurons and brainstem noradrenergic neurons (Calogero et al., 1988; Silverman, Hou-Yu and Chen, 1989). Control of this system also involves the mPFC that directly projects to the hypothalamus and exerts an inhibitory control on the HPA axis (Diorio, Viau and Meaney, 1993).

Catecholamines were for a long time only associated to rapid stress response. Nowadays epinephrine is also considered as a relevant contributor to stress-related disorders such as

cardiorespiratory, immune, tumorigenic, and psychiatric abnormalities (for review Wong et al., 2012).

Table 1: Behavioral and physical adaptation during acute stress
(adapted from Chrousos and Gold, 1992).

Behavioral adaptation: adaptive redirection of behavior	Physical adaptation: adaptive redirection of energy
Increased arousal and alertness	Oxygen and nutrients directed to the CNS and stressed body site(s)
Increased cognition, vigilance, and focused attention	Altered cardiovascular tone, increased blood pressure and heart rate
Euphoria (or dysphoria)	Increased respiratory rate
Heightened analgesia	Increased gluconeogenesis and lipolysis
Increased temperature	Detoxification from toxic products
Suppression of appetite and feeding behavior	Inhibition of growth and reproduction
Suppression of reproductive axis	Inhibition of digestion-stimulation of colonic motility
Containment of the stress response	Containment of the inflammatory/immune response

b) Monosynaptic hypothalamic inputs: energetic resources regulation

The arcuate nucleus of the hypothalamus also behaves as an energetic sensor (Levin, 2002). Circulating factors such as leptin (Ahima et al., 2000), glucose (Levin, 2002), and insulin (Belgardt, Okamura and Brüning, 2009) play a crucial role in the regulation of energy stores and neuroendocrine axes. Neurons of the arcuate nucleus project to the PVN to stimulate (*via* neuropeptide Y; Haas and George, 1987; Wahlestedt et al., 1987) or inhibit (*via* POMC secretagogues; Xiao et al., 2003; Cragnolini et al., 2004) stress-induced activation of the HPA axis.

1.1.2. Glucocorticoids and central modulation of the stress response

In addition to be involved in the prompt response to stress, glucocorticoids also mediate a long-term adaptation to stress. However, if stress occurs repeatedly and the physiological response becomes inadequate, excess of glucocorticoids may cause the development of stress-related disorders (Ulrich-Lai and Herman, 2009; McEwen and Gianaros, 2010). Glucocorticoids appear to be the final mediators of the stress response (Wong et al., 2012). There is little doubt that increased levels of glucocorticoid are a specific hallmark of a stressing state. For example,

rats exposed to a predator and people reporting high levels of stress consistently show higher plasma levels of glucocorticoids (corticosterone and cortisol, respectively) (Mesches et al., 1999; Wolf et al., 2001).

a) Glucocorticoids secretion: pathways of the hypothalamic-pituitary-adrenal axis

Physiologically, there is a circadian fluctuation of glucocorticoids secretion (Chung, Son and Kim, 2011). Increases in plasma glucocorticoids levels occur at the time of arousal to set the appropriate conditions for awake activities in both nocturnal and diurnal vertebrates (for review Kalsbeek et al., 2012). Oscillatory secretion of glucocorticoids is regulated by inhibitory projections of the suprachiasmatic nucleus (SCN) of the hypothalamus to the PVN (Szafaraczyk et al., 1983). In addition to the circadian pattern of glucocorticoids release, there is an ultradian pattern of discrete pulsatile release (Walker, Terry and Lightman, 2010), mainly occurring in humans, monkeys, and other mammals including rodents, characterized by discrete peaks occurring at approximately hourly intervals (Lightman et al., 2008). Increased circulating levels of glucocorticoids during awakening are believed to be essential for optimizing the functional tone of numerous physiological systems (for review DeKloet et al., 1998). In particular, glucocorticoids are critical regulators of brain function. The circadian patterns of molecules that regulate the HPA axis might be perturbed by environmental changes in lighting, feeding schedules and activity, as well as by stress.

The hypothalamus is the great conductor of the HPA axis hormonal orchestra (Fig. 3). The PVN of the hypothalamus includes parvocellular neurons, which synthesize and release CRH in the hypothalamo-hypophyseal portal system located in the ventral face of the median eminence (Antoni, 1986; Whitnall, Kiss and Aguilera, 1993). CRH and AVP produced by parvocellular and magnocellular neurons of the PVN act on corticotrophic cells of the anterior pituitary to induce the production of pro-opiomelanocortin (POMC). POMC is cleaved by prohormone convertase 1 and 2 and carboxypeptidase E into adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormones (MSHα, -β, and –γ), β-endorphin, and other bioactive peptides. ACTH released from the anterior pituitary targets the *zona fasciculata* and *zona reticulata* of the adrenal cortex where it binds to MC2 receptors to stimulate the production of glucocorticoids (cortisol in humans and corticosterone in rodents) and androgens. ACTH has a minor role in the *zona glomerulare* of the adrenal cortex, where potassium levels and angiotensin-II are the main regulators of mineralcorticoid production (Fig. 2 and 3).

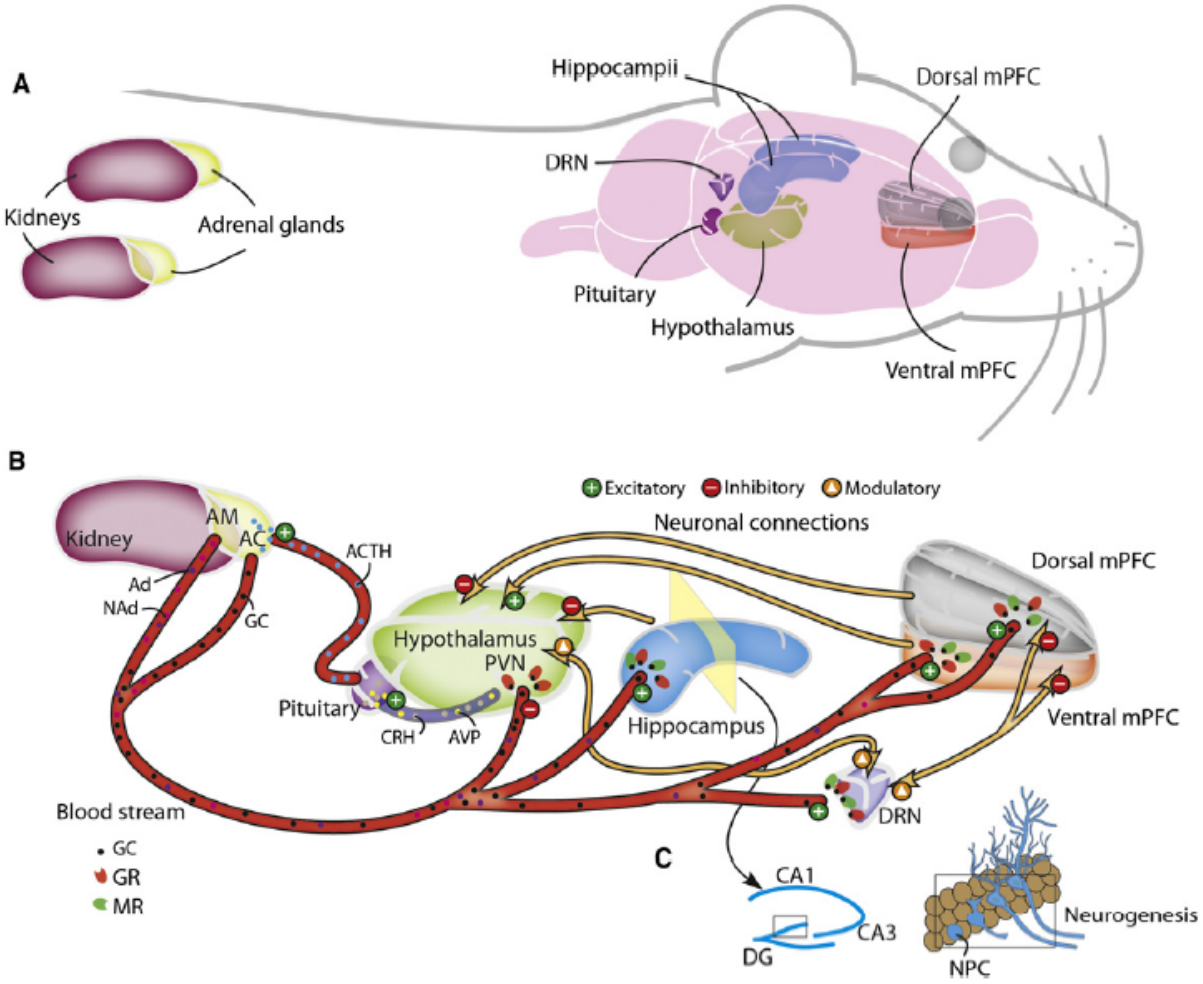

Figure 3: (A) Major components of the HPA axis and connected brain structures. (B) Connections between the hypothalamus, pituitary, and adrenal glands in the HPA axis, and hippocampus, medial prefrontal cortex (mPFC), and dorsal raphe nucleus (DRN).

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

Although CRH and AVP act synergistically in stimulating ACTH secretion, AVP has little secretagogue activity on its own (Gillies, Linton and Lowry, 1982; Abou-Samra et al., 1987). CRH appears to play a crucial role in ACTH secretion during acute stress, while AVP is necessary for the maintenance of basal ACTH levels (de Keyzer et al., 1997). Within the hypothalamus, there is a reciprocal interaction between AVP and CRH, which positively stimulate each other secretion. Under non-stressful conditions, secretion of both CRH and AVP in the portal circulation is circadian and pulsatile. This particular pattern of secretion is maintained along the entire HPA axis (Horrocks et al., 1990; Veldhuis et al., 1990; Calogero et al., 1992). When the organism is exposed to stress, neurons in the PVN synchronize to increase the pulsatile release of CRH and AVP in the circulation (Calogero et al., 1992). If stress becomes persistent, there is a marked shift in hypothalamic CRH/AVP signal in favor of AVP, as well as a down-regulation of CRH receptors within the anterior pituitary, suggesting a dynamic role for AVP in regulating the HPA axis (Scott and Dinan, 1998). In addition to neural pathways, depending on the nature of the stressors (internal and/or external changes), other circulating factors, such as angiotensin II (signaling dehydration; Lind, Swanson and Ganten, 1984; Plotsky et al., 1988), pro-inflammatory cytokines, and lipid mediators of inflammation contribute to the activation of the HPA axis (for review Charmandari, Tsigos and Chrousos, 2005; Herman, 2012).

Activation of the immune system is another phenomenon relevant for the multifaceted aspects of the stress response. While anti-inflammatory actions of glucocorticoids during stress was first believed to be needed in order to save energy for more critical needs, in recent years it has been proposed that glucocorticoids first exert a pro-inflammatory action (“permissive” effect) and subsequently an anti-inflammatory action to shut down the immune response and restore homeostasis (Busillo and Cidlowski, 2013). In the CNS, glucocorticoids exert a dual action, being both pro- and anti-inflammatory depending on the brain region as well as on the injury state (Sorrels and Saposly, 2007).

b) Indirect pathways regulating hypothalamic-pituitary-adrenal axis activity

Control of hypothalamic neurons activity implicates various integrative systems organized in polysynaptic pathways that regulate the activity of CRH neurons in the medialparvocellular PVN (mpPVN) of the hypothalamus (Fig. 4). This includes: *i*) the peri-PVN area that mainly consists of glutamatergic limbic system afferents (Ziegler and Herman, 2000) and γ-aminobutyric acid (GABA)-ergic interneurons directly projecting on CRH neurons of the PVN;

ii) intermediate areas formed by the bed nucleus of the stria terminalis (BNST), the medial preoptic area (POA) and the ventrolateral region of the dorsomedial hypothalamic nucleus (vlDMH) that sends GABAergic innervation; *iii*) cortico-limbic structures, e.g. the hippocampus and mPFC, which negatively regulate the activity of the HPA axis, as well as the central nucleus of the amygdala (CeA), which instead exerts a stimulatory control on the HPA axis. Brainstem nuclei, such as LC and *nucleus tractus solitarii* (NTS) are also involved in the regulation of the HPA axis (Herman et al., 2003; for review Herman, 2012). Infralimbic (il) cortices also play a role in the HPA response to stress, as shown by the evidence that lesions of these regions decrease glucocorticoid response to stress (Sullivan and Gratton, 1999; Radley, Arias and Sawchenko, 2006). Regulation of the stress response by il cortex relays in the anteroventral bed nucleus of the stria terminalis (BNST) that sends direct projections to the PVN (Dong et al., 2001). In addition to these indirect pathways, serotoninergic neurons from the dorsal raphe nucleus (DRN) also modulate the activity of the HPA axis (Fig. 3). Serotonin (5-HT) directly stimulates CRH neurons in the PVN of the hypothalamus through the activation of 5-HT_{2A} receptors (Zhang et al., 2002). The established role of the DRN in the regulation of mood and anxiety supports the general belief that dysfunction of the HPA axis are involved in the pathophysiology of psychiatric disorders.

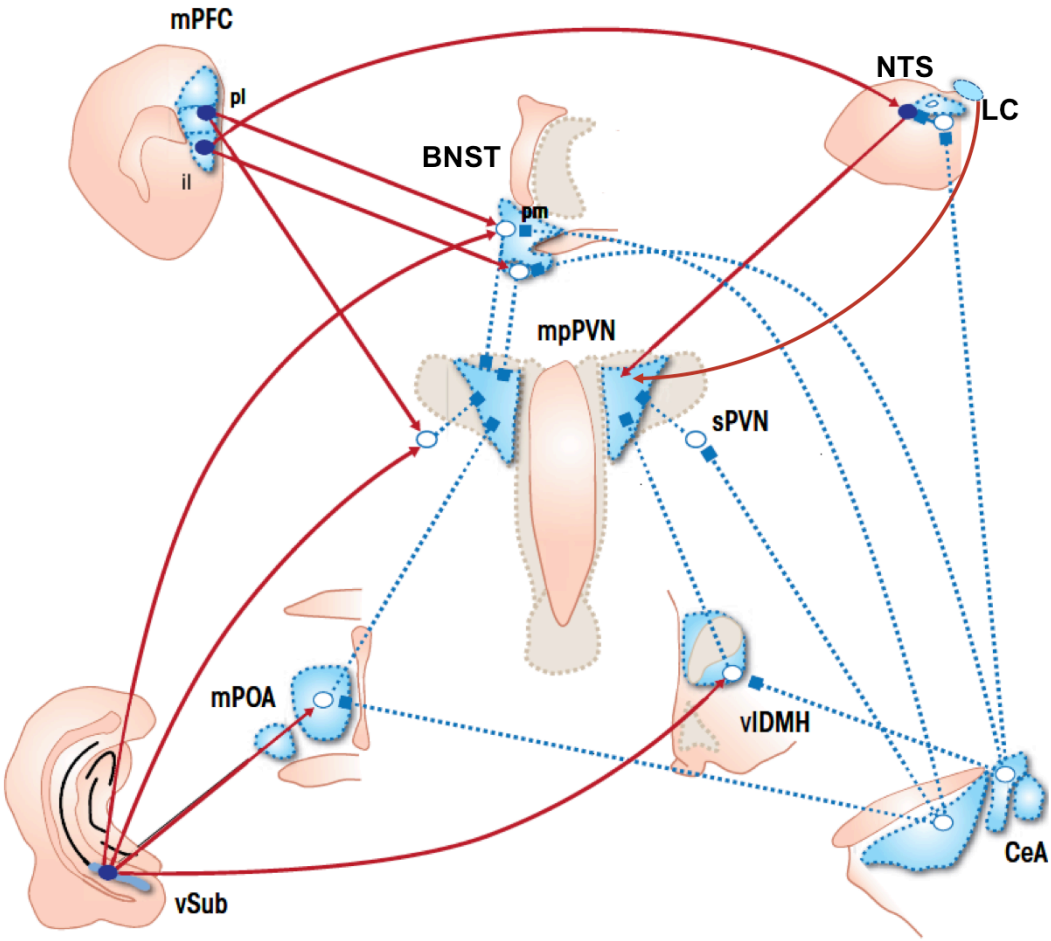

Figure 4: Schematic of limbic stress-integrative pathways from the prefrontal cortex, amygdala and hippocampus.

The medial prefrontal cortex (mPFC) subsumes neurons of the prelimbic (pl) and infralimbic cortices (il), which appear to have different actions on the hypothalmic–pituitary–adrenal (HPA) axis stress response. The pl sends excitatory projections (designated as darv circles, filled line with arrows) to regions such as the peri-PVN (peri-paraventricular nucleus) zone and bed nucleus of the stria terminalis (BNST), both of which send direct GABAergic projections to the medialparvocellular PVN (mpPVN, delineated as open circles, dotted lines ending in squares). This two-neuron chain is lively to be inhibitory in nature. In contrast, the infralimbic cortex projects to regions such as the nucleus of the solitary tract (NTS), the locus coeruleus (LC) and the anterior BNST, which sends excitatory projections to the PVN, implying a means of PVN excitation from this cortical region. The ventral subiculum (vSUB) sends excitatory projections to numerous subcortical regions, including the posterior BNST, peri-PVN region (including the subparaventricular zone [sPVN], medial preoptic area [POA] and ventrolateral region of the dorsomedial hypothalamic nucleus [vlDMH]), all of which send GABAergic projections to the PVN and are lively to communicate transsynaptic inhibition. The medial amygdaloid nucleus (MeA) sends inhibitory projections to GABAergic PVN-projecting populations, such as the BNST, POA and sPVN, eliciting a transsynaptic disinhibition. A similar arrangement lively exists for the central amygdaloid nucleus (CeA), which sends GABAergic outflow to the ventrolateral BNST and to a lesser extent, the vlDMH. The CeA also projects to GABAergic neurons in the NTS, which may disinhibit ascending projections to the PVN (adapted from Herman, 2012).

c) Non-genomic inhibition of the hypothalamic-pituitary-adrenal axis

Since the early 80's, one established function of glucocorticoids is to protect the organism against an excessive metabolic and functional response to stress (Munck, Guyre and Holbrook, 1984). Nevertheless, high levels of glucocorticoids might be detrimental to the organism. This highlights the importance of a finely tuned regulation of glucocorticoid secretion.

Glucocorticoids feeds-back to the pituitary gland to inhibit the production of ACTH (Keller-Wood and Dallman, 1984). ACTH in turn inhibits its own production, as well as the production of CRH and AVP in the PVN *via* a short negative feedback loop (Sawchenko and Arias, 1995). Glucocorticoids also act at hypothalamic level to reduce CRH secretion through the production of endocannabinoids that inhibit glutamate release at excitatory synapses (Di et al., 2003). At this level, the action of glucocorticoids is also mediated by the anti-inflammatory potein, annexin 1 (Buckingham et al., 2006). Most actions of glucocorticoids are mediated by their binding to cytosolic receptors (see below) that translocate to the nucleus acting as transcription factors. However, several lines of evidence indicate that glucocorticoids also produce rapid non-genomic effects that are mediated by membrane receptors. Some of the effects of glucocorticoids at the level of the PVN and the pituitary gland are ascribed to this mechanism (reviewed by Dallman, 2005; Fig. 2).

1.1.3. Glucocorticoids receptors

Glucocorticoids, the final actors of the stress response, exert their action through specific receptors that are ubiquitously distributed, and particularly expressed in the stress-integrative pathways (Reul and de Kloet, 1985; Funder and Sheppard, 1987). In 1985, Reul and de Kloet showed the existence of two receptors system for corticosteroids: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). Both receptors are activated by glucocorticoids, but only MRs interact with mineralocorticoids (e.g., aldosterone in humans). In spite of their name, MRs display higher affinity for glucocorticoids than GRs. Thus, MRs correspond to type-I high affinity glucocorticoid receptors, whereas GRs correspond to type-II low affinity glucocorticoid receptors (Reul and de Kloet, 1985; Arriza et al., 1988). GRs have been extensively investigated in the last several years, and the molecular determinants of their function have been fully disclosed. The GR, which is also named NR3C1 (nuclear receptor subfamily 3, group C, member 1) plays a major role in the anti-inflammatory/immune suppressant and metabolic action of glucocorticoids and is also involved in the feedback regulation of the HPA axis in response to high levels of glucocorticoids (e.g., under stress conditions). The GR protein contains different functional domain, of which domain I (AF1) contains a trans-activating

sequence, domain II contains a zinc-finger sequence that binds to responsive element in the DNA, domain III is a hinge followed by domain IV, which contains the glucocorticoid binding site, the dimerization domain, the nuclear translocation signal, and an additional trans-activation domain (AF2). Unbound GR is present in the cytosol where is linked to molecular chaperones, such as heat-shock protein (Hsp)-70 and -90, FK-506 binding protein-type 12 (FKBP12), p23, and Src (reviewed by Pratt et al., 2006). Binding to glucocorticoids causes the nuclear translocation of GRs, which, in form of dimers bind to glucocorticoid-responsive elements (GREs) activating or repressing gene expression (Beato, 1989). Positive GREs are formed by a palindromic sequence (TGTACAnnnTGTt/cCT). For example, genes encoding for enzymes of gluconeogenesis are induced by the interaction of GRs with positive GRE. Negative GREs (GGAAGGTCACGTCCA) mediate the negative regulation of POMC and CRH expression by glucocorticoids (Drouin et al., 1993) If GREs are distant from the TATA box (the site of start of gene transcription), the glucocorticoid/GR/GRE complex recruits co-activators or co-repressors (e.g., histone-acetyl transferases and histone deacetylases, respectively), which shape the gene response to glucocorticoids. Interestingly, *O*-GlcNAc transferase (OGT), the enzyme that catalyzes addition of *O*-N-acetylglucosamine (*O*-GlcNAc) moiety to substrate proteins has been implicated in mechanisms of glucocorticoid receptor-mediated transrepression (Li et al., 2012). The glucocorticoid-GR complex may also interact with other transcription factors, such as AP1 (formed by dimers of Fos and Jun proteins) or NFκB (de Kloet et al., 1998; Webster and Cidlowski, 1999).

Very recently, Polman, de Kloet and Datson (2013) identified two populations of GREs (named "GR-binding sites" or GBS) that are recruited as a function of glucocorticoid levels. The GBS that is recruited in response to a wide range of glucocorticoid levels regulates the expression of circadian genes, whereas the GBS specifically recruited in response to high levels of glucocorticoids regulates the expression of genes involved in neuronal plasticity. According to De Kloet (2013), the GBS responding to a wide range of glucocorticoid might mediate the permissive effect of corticosterone during the circadian cycle, whereas the low affinity/high capacity GBS might be relevant to stress regulations.

a) Function of mineralocorticoid receptors

MRs are characterized by high affinity for glucocorticoids but low selectivity, recognizing aldosterone as a ligand. They are highly saturated by low resting levels of glucocorticoids and play a major role in the feedback regulation of the HPA axis under normal, non-stressfull,

conditions (Bradbury, Akana and Dallman, 1994; de Kloet et al., 1998). Thus, MRs are involved in the tonic effects of glucocorticoids (de Kloet and Reul, 1987). Peripherally, MRs are distributed in aldosterone target tissue (i.e. distal nephron of the kidney, colon, sweat and salivary glands, cardiovascular system), where they are mainly involved in electrolytic homeostasis (Farman and Rafesin-Oblin, 2001). In the kidney, glucocorticoids cannot activate MRs because they are metabolized by type-2 11-β-hydroxysteroid dehydrogenase (11β−HSD2; see below). In the CNS, MRs are expressed in the hippocampus, amygdala (van Eekelen et al., 1988; van Steensel et al., 1996), hypothalamic nuclei (Reul and de Kloet, 1985; van Eekelen, Bohn and de Kloet, 1991). MRs are also expressed in the pituitary gland. In general, the distribution of MRs is more restricted than the distribution of GRs.

b) Functions of glucocorticoid receptors

GRs have a lower affinity for corticosterone than MRs (their K_D is five to ten fold higher then the K_D of MRs; Reul and de Kloet, 1985), but their selectivity is higher. GRs are recruited by high circulating levels of glucocorticoids, which are found in response to stress (Reul and de Kloet, 1985; de Kloet et al., 1998). GRs mediate phasic effects of glucocorticoids (de Kloet and Reul, 1987). Both MRs and GRs are co-localized in limbic areas (van Eekelen et al., 1988; van Steensel et al., 1996), although GR distribution is ubiquitous. Particularly, GRs are expressed in the hippocampus, amygdala, lateral septum, cortex, LC, NTS, hypothalamic PVN, and anterior pituitary (Reul and de Kloet, 1985), all structures that are involved in the regulation of stress response. As outlined above, GRs play a major role in the feedback regulation of the HPA axis under conditions of stress (de Kloet and Reul, 1987). Accordingly, studies carried out in GR-knockout mice show that this receptor is required for the extinction of HPA activity in response to stress (Boyle et al., 2005; Furay, Bruestle and Herman, 2008).

Regulation of HPA activity by glucocorticoids takes place at multiple levels. This enables an efficient response to stress avoiding excessive detrimental consequences. Key sites of action include the hippocampus, PVN and pituitary (Fig. 2). Binding of glucocorticoids to GR in the hippocampus results into enhanced inhibitory signals to the PVN with ensuing reduction of the HPA activity (Joëls and de Kloet, 1993). Downstream in the regulatory pathway of the HPA axis, glucocorticoid negative feedback is mediated by GR activation in the PVN and anterior pituitary. In this particular case, GRs act as transcriptional repressors by inhibiting CRH and POMC gene expression (Drouin et al., 1993; Martens et al., 2005).

1.1.4. Local regulation of glucocorticoid bioavailability

Following type-2 melanocortin receptors (MC2 receptors) activation by ACTH in the adrenal cortex, cholesterol traslocates into the mitochondria, where is transformed into pregnenolone by the enzyme CYP11A1. Pregnenolone is then converted by 3β-hydroxysteroiddehydrogenease-Δ-4,5-isomerase into progesterone, which is then transformed into 17α-hydroxyprogesterone by CYP17, which is a selective marker of the *zona reticulata* and *zona fasciculata* of the adrenal cortex. CYP21 transforms 17α-hydroxyprogesterone into 11-deoxycortisol, which is then converted into cortisol by CYP11B1. Regulatory system exists to readily maintain circulating levels of glucocorticoids that reach target tissues.

a) Corticosteroid binding protein

Due to their lipophilic properties circulating glucocorticoids reversibly bind to plasma proteins such as albumin (15%) and, more specifically, corticosteroid binding protein (CBG, or transcortin, 80%). As suggested by the free hormone hypothesis, only the 5% free fraction is able to enter the brain and reach other target tissues (Mendel, 1989).

CBG is a glycoprotein synthesized in the liver and released into the bloodstream where it binds glucocorticoids with high affinity but low capacity, as opposed to albumin that displays low affinity but high capacity (for review Moisan et al., 2013). CBG critically regulates the access of adrenal steroids to target tissues and appears to have a dual role acting both as a buffer, locally delivering glucocorticoids to their target cells, and as a reservoir of glucocorticoids in the blood (Hammond, 1995; Breuner and Orchinik, 2002). In response to stress, CBG buffers the rise of free glucocorticoids by sequestering them into an inactive complex. However, this capacity is restrained by the lowering effect of stress on CBG levels (Fleshner et al., 1995). Nowadays, CBG is more considered as a regulator of glucocorticoids levels maintaining circulating pool of glucocorticoids and delivering them to target tissues (for review Moisan et al., 2013).

b) 11β-hydroxysteroid-dehydrogenases

An additional level of control is exerted by tissue-specific enzymes, which locally metabolize glucocorticoids. In their free biologically active form, glucocorticoids are chemically 11-hydroxy-glucocorticoids (e.g., cortisol, corticosterone), while their inert forms correspond to 11-keto-metabolites (cortisone and 11-dehydrocorticosterone, respectively). 11β-HSD2 converts cortisol and corticosterone into inactive cortisone and 11-dehydrocorticosterone,

whereas 11β-HSD1 catalizes the opposite reaction (Amelung et al., 1953; Seckl and Chapman, 1997; reviewed by Seckl and Walker, 2001).

11β-HSD1 is widely expressed, most notably in liver, lung, adipose tissue, vasculature, ovary, and the CNS, where it colocalizes with GRs (Monder and White, 1993; Stewart and Krozowski, 1999). This enzyme has a nicotinamide adenine dinucleotide phosphate (NADP(H))-dependent activity and mainly acts as a reductase to reactivate glucocorticoids, i.e. 11β-HSD1 catalyzes the 11β-reduction regenerating active forms of glucocorticoids from inert keto-products (Lakshmi and Monder, 1988; Jamieson et al., 2000). Locally, 11β-HSD1 amplifies glucocorticoids levels (reviewed by Seckl and Walker, 2001; Yau and Seckl, 2012). This isoform is also predominant in the adult rodent and human brain, where it is widely distributed with particularly high expression in the hippocampus, PVN of the hypothalamus, pituitary, cerebellum, and cortex in both neurons and glial cells (Moisan et al., 1990; Harris et al., 2001; Sandeep et al., 2004). Because of its expression in key regions of the HPA axis, 11β-HSD1 may influence the feedback regulation of the stress response (Seckl, 1997).

As opposed to 11β-HSD1, 11β-HSD2 is a high-affinity NAD-dependent dehydrogenase, which exclusively catalyzes the inactivation of physiological glucocorticoids (Seckl and Walker, 2004). This enzyme colocalizes with MRs in distal nephron, colon, sweat gland, testes and placenta, where 11β-HSD2 restrains the activation of MRs by glucocorticoids (Stewart and Krozowski, 1999). Expression of 11β-HSD2 in the kidney limits the potential sodium-retentive action of cortisol and corticosterone, thus avoiding large increases in blood sodium concentrations and plasma volume under stress conditions. Interestingly, this protective function of 11β-HSD2 is reduced by glycirrhizinic acid, a licorice component that increases blood pressure (Edwards et al., 1988; Funder et al., 1988).

1.1.5. Involvement of oxytocin in the regulation of the hypothalamic-pituitary-adrenal axis

As discussed above, the PVN of the hypothalamus is a crucial node in the regulation of the HPA axis. The PVN is the crossroad for signals that regulate many facets of the response to stress, such as the HPA axis itself and the autonomic nervous system, and the cognitive and emotional responses to stress. Neurohypophyseal hormones secreted by the PNV, i.e., AVP and oxytocin, are involved in most of these functions (Swanson and Sawchenko, 1983). Oxytocin and AVP are synthesized in magnocellular neurons of the PVN and the supraoptic nucleus (SON) of the hypothalamus that project through the median eminence to the posterior pituitary,

where both hormones are released into the systemic circulation in response to specific stimuli (for review Cunningham and Sawchenko, 1991). The existence of a connection between the SON and the posterior pituitary has been demonstrated in the 19th century by Ramón y Cajal (1894), who was the first to describe a neural pathway from the SON to the posterior pituitary. In 1951, Wolfgang Bargmann and Ernst Scharrer showed that AVP and oxytocin are stored and secreted in the posterior pituitary.

Because oxytocin and AVP only differ in two amino acids (oxytocin: NH_2-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-COOH; AVP: NH_2-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-COOH), it is not surprising that they share several functional properties (Lippert et al., 2003). Besides their role in numerous social behaviors (de Wied, Diamant and Fodor, 1993), both hormones are involved in the regulation of stress response (Engelmann, Landgraf and Wotjak, 2004). There is evidence that magnocellular PVN neurons are activated in response to stress (Gibbs et al., 1984). However, the specific role of oxytocin and AVP in stress response was recognized several years after their main actions (ecbolic and antidiuretic/vasopressor effects, respectively) had been described (du Vigneaud et al., 1954; Acher, Light and du Vigneaud, 1958). Of note, oxytocin was named by Sir Henry Dale from the Greek words "ωκνξ" and "τοκοξ", i.e. "swift birth", when he discovered in 1906 that extracts of human posterior pituitary gland contracted uterus of pregnant cat.

Gibbs et al. (1984) have demonstrated that oxytocin acts as a secretagogue of ACTH in the anterior pituitary (Gibbs et al., 1984). This oxytocin-induced ACTH secretion is suppressed by glucocorticoids. In the early 1990s, Link and colleagues (1992) demonstrated that physiological concentrations of oxytocin, applied in a pulsatile fashion, induce ACTH release in isolated anterior pituitary cells. While increased levels of AVP are mainly observed in stress related to osmotic changes, physical stressors, such as immobilization, induce an enhancement in circulating oxytocin levels (Lang et al., 1983; Jezova et al., 1995). Increases in oxytocin levels are also observed under conditions of chronic stress (Kiss and Aguilera, 1993; Glasgow et al., 2000), but highly depends on the type of stressor (for review McQuaid et al., 2014). Interestingly, within the hypothalamic-hypophyseal system, oxytocin and CRH produce reciprocal effects. CRH stimulates oxytocin release (in addition to ACTH as reported above; Bruhn et al., 1986) and genetic deletion or pharmacologic inhibition of CRHR1 reduces stress-induced corticosterone response as well as oxytocin production (Müller et al., 2000; Keck et al., 2003). Also, restraint stressors induce a substantial increase in CRH mRNA levels in oxytocin knockout (KO) mice (Nomura et al., 2003), while, on the other hand, injection of

oxytocin attenuates the rise in CRH mRNA levels in rats exposed to acute restraint stress (Bülbül et al., 2011). Dabrowska and colleagues (2011) recently showed the existence of a potential feedback loop between the hypothalamic oxytocin system and the forebrain CRH system. Among the putative molecular players of the stress axis, norepinephrine is also involved in the regulation of oxytocin secretion (reviewed by McQuaid et al., 2014), adding another level of complexity to the multifaceted regulation of the stress response. Neurophypohiseal hormone might also be involved in the immune response to stress by displaying a cytokine-like action (reviewed by Herman, Flak and Jankord, 2008).

On the other hand, oxytocin negatively controls ACTH and corticosterone secretion and displays anxiolytic properties (Windle et al., 1997a; 2004; Neumann et al., 2000). A large body of evidence demonstrates that acute oxytocin administration has a stress-protective role in rodents (Bale et al., 2001; Jurek et al., 2012) and humans (Guastella et al., 2009; Neumann and Landgraf, 2012). Consistent with the inhibitory role of oxytocin on the HPA axis, Amico and colleagues (2008) showed that deletion of the oxytocin gene in mice heightens the corticosterone response to stress. Remarkably, the stress response has been found to be decreased under conditions in which circulating levels of oxytocin are high, e.g., in lactating dams (Walker et al., 1992). Of note, under conditions of chronic stress, low doses of oxytocin locally infused in the hippocampus produce beneficial effects, which are lost with higher doses (Peters et al., 2014). This suggests that the impact of oxytocin on the HPA axis and, more in general, on the brain response to stress is not an all-or-none phenomenon, but it critically depends on the amount of oxytocin secreted inside the brain or in the peripheral circulation.

1.2. Hippocampal glutamatergic transmission and stress response

Corticolimbic structures such as the hippocampus, amygdala, and prefrontal cortex are highly involved in the regulation of the stress response and are targeted by the target for stress-related hormones, such as glucocorticoids (Herman et al., 2003). Cortico-limbic circuits mediate the behavioral response to stress (for review Maren and Quirk, 2004; McLaughlin, Baran and Conrad, 2009). In addition, the hippocampus, amygdala and prefrontal cortex play a major role in the regulation of mood and emotions, and are therefore implicated in the pathophysiology of stress-related mental disorders (Phillips et al., 2003; Radley et al., 2015).

Some of the experimental data reported below will deal with glutamate release in the hippocampus. Hence, a short synopsis on the anatomical organization of the hippocampus, the

basic features of glutamatergic neurotransmission and the role for glutamate in stress-related disorders will follow.

1.2.1. Anatomical organization of the hippocampus

The hippocampus plays a key role in restraining activation of the HPA axis (Bohus, 1975). Hippocampal activation reduces glucocorticoid secretion from the adrenals (Dunn and Orr, 1984), while hippocampal lesions produce the opposite effect both under basal conditions and in response to stress (Herman et al., 1989). Inhibition of the HPA axis is mediated by the ventral subiculum (vSUB; Herman et al., 2005), and is instrumental for homeostatic regulation of the HPA axis after stress. Excitatory axons of hippocampal pyramidal neurons project to regions that negatively modulate the activity of PVN hypothalamic neurons, such as the posterior BNST, and the peri-PVN regions including the subparaventricular zone (sPVN), the medial preoptic area (POA), and the vlDMH. These regions send inhibitory GABAergic projections to the PVN (Fig. 4; Herman, 2012). The hippocampus is enriched in both MRs and GRs, which mediate the feedback regulation of the HPA axis by glucocorticoids under basal conditions and in response to stress, respectively (Reul and de Kloet, 1985; van Eekelen et al., 1988; Seckl et al., 1991; Reul et al. 2000a; b; Barbaccia et al., 2001).

Lorente de No (1934) has subdivided the hippocampus into different subfields that separate the dentate gyrus (DG) from the subiculum: the CA1 (cornu Ammonis 1, *regio superior*, or Sommer's sector), CA2, CA3 (*regio inferior*), and CA4. These subfields are interconnected through specific unidirectional excitatory circuit (Fig. 5), which includes (*i*) the mossy fibers (MF), which are the axons of DG granule cells projecting to CA3 pyramidal neurons; and (*ii*) the Schaffer collaterals (SC), which are the axon of CA3 pyramidal neurons projecting to the CA1 region. The DG, all CA subfields, and the subiculum all receive excitatory afferents from the entorhinal cortex (the perforant pathways). Fibers originating from layers II and VI of the entorhinal cortex project to the DG and CA3 region, whereas fibers originating from layers III and V (which form the "temporoammonic pathway") project to the CA1 region and the subiculum. Each subregion of the hippocampus responds to stress and is subjected to morphological and functional modifications under conditions of chronic stress (Wellman, 2011).

The hippocampus plays a key role in cognitive and emotional processes. Since the groundbreaking case of patient H.M., who lost much of his memory after a temporal lobectomy (a surgery performed for the treatment of his intractable epilepsy), a vast amount of data has

linked the hippocampus to memory in humans, other primates, rats and mice (Scoville & Milner, 1957; Squire, 1992). In the late 90's, Maguire and colleagues (1997) highlighted the specific role of the hippocampus in spatial memory studying activation of this structure in London taxi drivers while they were remembering a specific itinerary. Its specific role in food catching behavior has also been investigated in little animals (Biegler et al., 2001; Burger et al., 2013). Moreover, lesion of this structure induces impairment in mnesic abilities in rodents (Ergorul and Eichenbaum, 2004). While the dorsal part of the hippocampus is specifically involved in memory function, the ventral part modulates emotional and affective processes (Fanselow and Dong, 2010). In particular, the ventral portion of the hippocampus is a key station of the stress neuronal circuit and is tightly connected to brain regions that are involved in the regulation of mood, anxiety, and social behavior, such as the ventral tegmental area, nucleus accumbens, amygdala, and medial prefrontal cortex (Belujon and Grace, 2011). Thus, this structure is also tightly associated to emotional behavior.

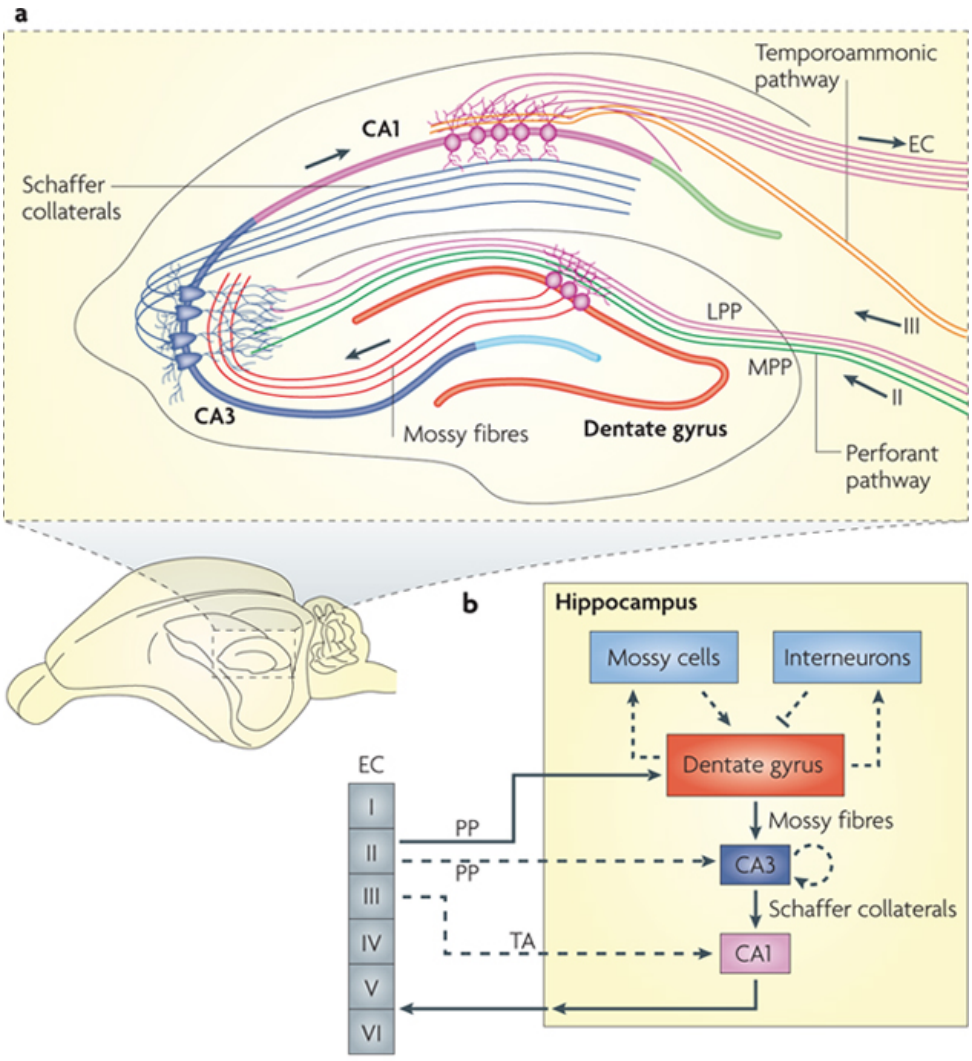

Figure 5: (A) The hippocampal circuitry. (B) Diagram of the hippocampal neural network.
The traditional excitatory trisynaptic pathway (entorhinal cortex (EC)–dentate gyrus–CA3–CA1–EC) is depicted by solid arrows. The axons of layer II neurons in the entorhinal cortex project to the dentate gyrus through the perforant pathway (PP), including the lateral perforant pathway (LPP) and medial perforant pathway (MPP). The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibers. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons through the PP. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway (TA). The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells (from Deng, Aimone and Gage, 2010).

1.2.2. Basic features of the glutamatergic transmission

a) Glutamatergic synapses machinery

Glutamate is synthesized in neurons *via* different metabolic pathways, including desamination of the glutamine originating in glial cells (Erecińska and Silver, 1990). The established role of glutamine as a major precursor of glutamate laid the groundwork for spectroscopic measurement of brain glutamine levels as specific indicators of the glutamate used for neurotransmission. Glutamate synthesis in neurons and glial cells accounts for up to 20% of brain glucose utilization (Hertz, 2006). Glutamate can be metabolized back into glutamine in astrocytes (the so-called glutamate-glutamine cycle) and can also be decarboxylated into GABA by the enzyme, glutamate decarboxylase (GAD) (reviewed by Bak, Schousboe, and Waagepetersen, 2006; Fig. 6).

Three vesicular glutamate transporters (VGLUT) have been identified: VGLUT1 (Ni et al., 1994), which is heavily expressed in in the cerebral cortex, hippocampus and cerebellum, and is specifically found in glutamatergic terminals; VGLUT2 (Aihara et al., 2000), which is enriched in subcortical regions; and VGLUT3 (Gras et al., 2002), which is primarily found in non-glutamatergic neurons (e.g. GABA interneurons of the hippocampus). These vesicular transporters are characterized by (*i*) millimolar affinity for glutamate; (*ii*) an inability to transport aspartate, glutamine or GABA; (*iii*) a dependence on the proton gradient; (*iv*) a dependence on the vesicular transmembrane potential component of the pH gradient; and (*v*) a biphasic chloride dependence (reviewed by El-Mestikawy et al., 2011).

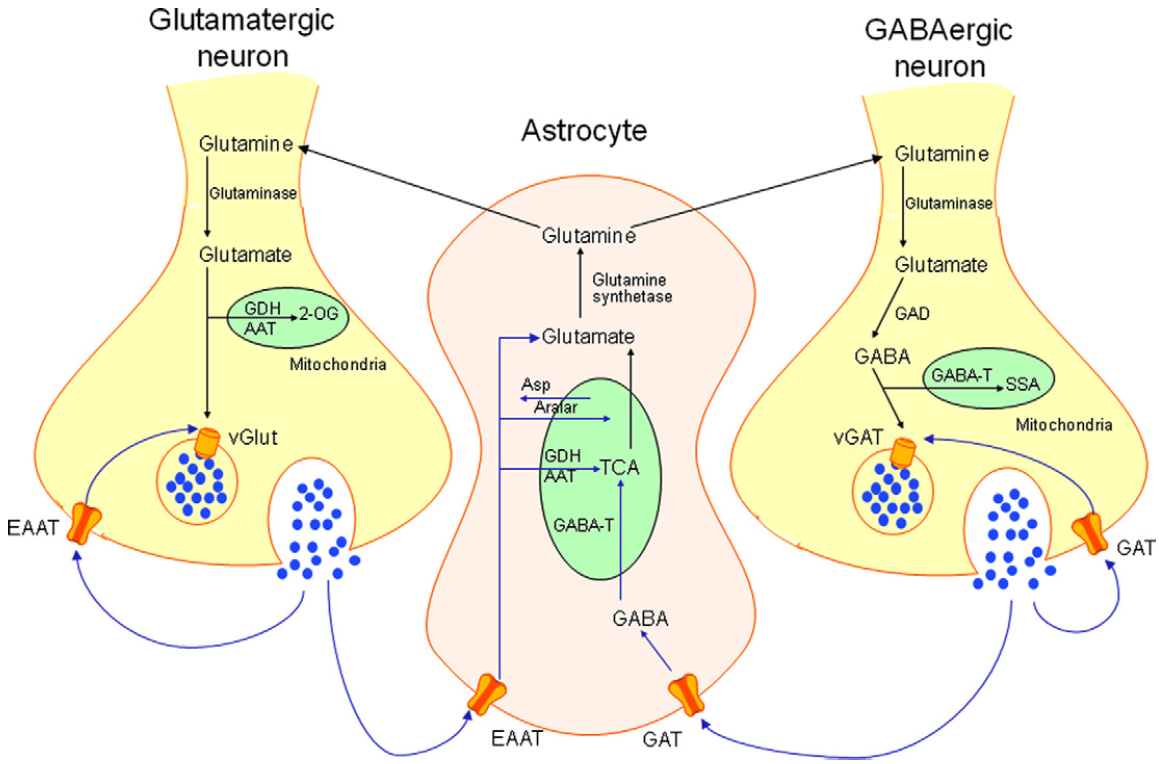

Figure 6: Cartoon summarizing the synthesis, packaging, release, transport, and metabolism of glutamate and GABA.

Glutaminase converts glutamine to glutamate, which is converted to GABA by GAD in GABAergic neurons. The neurotransmitters are subsequently transported by vesicular transporters into vesicles for release. Upon release, neurotransmitters are taken up by high affinity membrane transporters into neurons and surrounding glia where they can be recycled or metabolized via several enzymes.
2-OG, 2-oxoglutarate; AAT, aspartate aminotransferase; Aralar, aspartate-glutamate carrier; Asp, aspartate; EAAT, excitatory amino acid transporter; GABA-T, GABA transaminase; GAD, glutamic acid decarboxylase; GAT, GABA transporter; GDH, glutamate dehydrogenase; SSA, succinic semialdehyde; TCA, tricarboxylic acid cycle; vGAT, vesicular GABA transporter; vGlut, vesicular glutamate transporter (from Rowley et al., 2012).

Proteins of the SNARE complex (Soluble N-ethylmaleimide-sensitive factor Attachment Protein REceptor) (Fig. 7) allow vesicular glutamate release from presynaptic terminals. Synaptobrevins 1 and 2 (also called vesicle-associated membrane proteins or VAMPs) are the main vesicular SNARE (v-SNARE) proteins, whereas syntaxin 1 and 2, and SNAP25 are target membrane SNARE proteins (t-SNARE; Rothman, 1996). Munc18, which controls SNARE-complex formation and has also a direct role in synaptic vesicle membrane fusion, is specifically found in glutamatergic terminals (Lang and Jahn, 2008; Rizo and Rosenmund, 2008; Südhof and Rothman, 2009; Han et al., 2010; Fang and Lindau, 2014). Another specific marker of glutamatergic terminals is the monomeric GTP-binding protein, Rab3A, the most abundant and widely distributed Rab isoform in the brain, which plays a key role in glutamate

release and synaptic plasticity by regulating a late step in synaptic vesicle trafficking and fusion (Südhof, 2004; Takai Sasaki and Matozaki, 2001; Zerial and McBride, 2001). Synaptophysins are also involved in the regulation of vesicular trafficking in glutamatergic terminals.

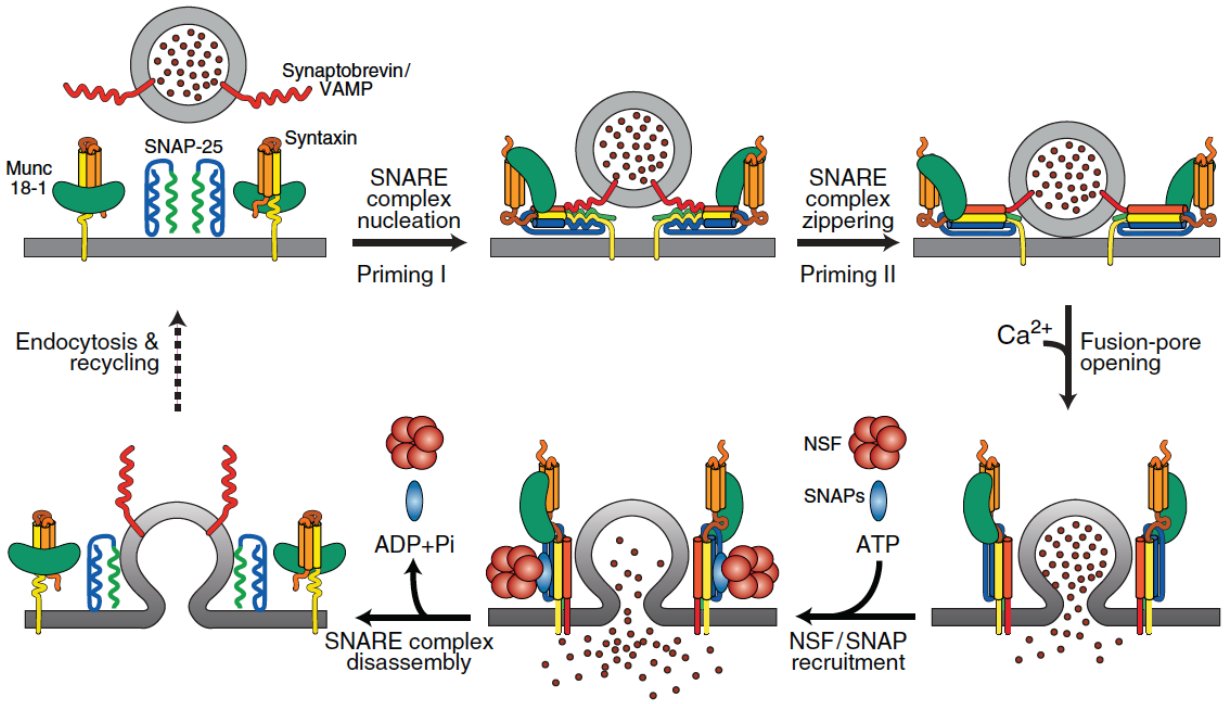

Figure 7: Schematic diagram of the SNARE protein/Munc18 cycle.
Docked synaptic vesicles (top left) may be attached to the active zone via the Rab/RIM interaction but contain SNARE proteins that have not yet formed a complex with each other (synaptobrevin/VAMP on synaptic vesicles and SNAP-25 and syntaxin-1 on the plasma membrane; note that syntaxin-1 is thought to be complexed to the SM-protein Munc18-1). Priming is envisioned to occur in two steps that involve the successive assembly of SNARE-complexes (priming I and II). During priming, Munc18-1 is thought to be continuously associated with syntaxin-1, shifting from a heterodimeric binding mode in which it was attached to syntaxin-1 alone to a heteromultimeric binding mode in which it is attached to the entire SNARE complex (top right). After priming, Ca2+ triggers fusion-pore opening to release the neurotransmitters by binding to synaptotagmin. After fusion-pore opening, SNAPs and NSF (an ATPase) bind to the assembled SNARE complexes, disassemble them with ATP-hydrolysis, thereby allowing synaptic vesicles to undergo re-endocytosis and to recycle with synaptobrevin on the vesicle, while leaving SNAP-25 and syntaxin-1/Munc18-1 on the plasma membrane. Note that the overall effect is that SNARE/Munc18-proteins undergo a cycle of association/dissociation that fuels the membrane fusion reaction, which underlies release (from Südhof, 2008).

A variety of membrane high affinity transporters (Gegelashvili and Schousboe, 1997) critically regulate the concentration of synaptic glutamate and their expression/activity is essential for the termination of glutamatergic signaling (because no glutamate-specific degradation enzymes are present at the synapse; O'Shea, 2002), and to limit the recruitment of potentially harmful

extrasynaptic glutamate receptors (e.g., the GluN2B-containing N-methyl-D-aspartate (NMDA) receptors). To date, five glutamate transporters have been identified and named excitatory amino acid transporters (EAAT)1 to -5. EAAT1 and -2 (GLAST and GLT-1, respectively) are present in astrocytes, whereas EAAT3 (EAAC1) and -4 are present in neurons (reviewed by Danbolt, 2001). Some of these transporters, e.g., GLT-1, are targeted by drugs of potential use in the treatment of chronic pain or neurodegenerative disorders (e.g., ceftriaxone in amyotrophic lateral sclerosis). Expression of glutamate transporters is not restricted to neurons or astrocytes, and has been documented in peripheral organs, such as the heart, liver, kidney and gut (Lerner, 1987). One transporter that does not fit into the above classification, called the cystine-glutamate antiport or Xc^- shows a widespread distribution in the organism, and exchanges intracellular glutamate with extracellular cystine. Xc^- present in astrocyte membranes releases the glutamate that may have access to receptors that are distant from the active site of neurotransmitter release, such as the mGlu2 metabotropic glutamate receptors. Changes in the expression of Xc^- have been linked to mechanisms of maladaptive synaptic plasticity associated with drug addiction (Reissner and Kalivas, 2010).

b) Glutamate receptors

There are two major categories of glutamate receptors: ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels, and metabotropic glutamate receptors (mGluRs), which are coupled to G proteins. iGluRs include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), NMDA, and kainate receptors. Changes in the expression, synaptic distribution, phosphorylation state, or subunit composition of AMPA receptors are associated with, and responsible for, changes in synaptic efficiency underlying activity-dependent forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD). At most synapses, NMDA receptors are silent under resting conditions but become active in response to membrane depolarization that are sufficient to remove the Mg^{2+} block of the ion channel. Because of this peculiar property, the NMDA receptor mediates the induction phase of both LTP and LTD, which requires the convergence of presynaptic glutamate release and postsynaptic depolarization (reviewed by Contractor and Heinemann, 2002; Kuner, Seeburg and Guy, 2003; Karakas, Regan and Furukawa, 2015; Zhu and Paoletti, 2015). Of note, *O*-GlcNAc of synaptic proteins and iGluRs (GluA2 AMPA subunit in particular) has been shown to be a modulator of hippocampal synaptic plasticity (Tallent et al., 2009; Kanno et al., 2010; Taylor et al., 2014). Excessive activation of NMDA receptors causes excitotoxic neuronal death, a mechanism that has been implicated in the pathophysiology of

acute and chronic neurodegenerative disorders. Excitotoxicity is mainly mediated by activation of GluN2B-containing NMDA receptors, which, at least in the adult life, are localized in the peripheral portion of dendritic spines. NMDA receptors antagonists have been developed for the treatment of neurodegenerative disorders in the last 25 years, with little or no success. The function of kainate receptors (formed by the GluK1 to -5 subunits) still needs to be fully clarified, although the evidence that kainate caused seizures in children and neuronal death in experimental animals was pioneering in the field of glutamatergic transmission.

mGluRs have been discovered in 1985 when glutamate and quisqualate were found to stimulate inositol phospholipid hydrolysis in cultured striatal neurons (Sladeczek et al., 1985), then in cerebellar granule cells (Nicoletti et al., 1986). Since that time the field has expanded dramatically, and drugs specifically targeting individual mGluR subtypes are currently under development for a variety of neurological and psychiatric disorders (Conn and Pin, 1997; Nicoletti et al., 2011; 2015). mGluRs form a family of eight subtypes, which are divided into three groups on the basis of their amino acid sequence, pharmacological profile, and transduction pathways. mGluR1 and mGluR5 are coupled to Gq/11, whereas group-II and group-III mGluR subtypes (mGlu2/3, and mGlu4/6/7/8, respectively) are all coupled to Gi/Go in heterologous expression systems (Conn et al., 2005; Swanson et al., 2005; Nicoletti et al., 2011). Glutamate and other orthosteric agonists bind to a bilobate Venus fly trap (VFT) domain, which is linked to the 7TM domain through a cystein-rich domain. This facilitates the closure of the two lobes, resulting into receptor activation (reviewed by Rondard and Pin, 2015). The characterization of the crystal structure of the 7TM regions of mGluR1 and mGluR5 bound to a negative modulator has been a recent breakthrough in the mGlu field (Doré et al., 2014; Wu et al., 2014). These findings will facilitate the development of subtype-selective positive and negative allosteric modulators of mGluRs, which are promising candidiates of CNS disorders (Conn et al., 2014).

c) Role of glutamate receptors in stress response and stress-related disorders

Stress-related disorders are believed to have in common an inappropriate or excessive synaptic excitability within crucial brain circuits, which causes a spectrum of psychiatric symptoms (Swanson et al., 2005). Stress-induced enhancement of glucocorticoid levels increases glutamate in the hippocampus (Lowy, Gault and Yamamoto, 1993; Stein-Behrens, Lin and Sapolsky, 1994; Abrahám et al., 1998). Animal studies suggest that both acute and chronic stress enhance in glutamate release. Accordinlgy, exposure of rats to acute stress induced a transient increase in extracellular glutamate levels in the prefrontal cortex (PFC; Moghaddam, 1993). Modulation of glutamatergic neurotransmission in the PFC after stress exposure may be aimed at facilitating executive functions and learning in order to orchestrate the adapted behavioral response (Holmes and Wellman, 2009). Increases in extracellular glutamate have also been observed in the CA1 region of the hippocampus following peripheral corticosterone administration (Venero and Borrell, 1999). Chronic exposure to restraint stress also induced similar increases in glutamate release in the hippocampus (Zhu et al., 2008).

Stress-induced increase in glutamate release may involve several mechanisms (Fig. 8). Treccani and colleagues (2014) showed that *in vitro* corticosterone application as well as acute stress increases the size of the readily releasable pool (RRP) of synaptic vesicles in the prefrontal cortex. Regulation of the RRP is strongly associated with the abundance of SNARE complex formation (Lonart and Südhof, 2010). Within the presynaptic terminal, Maurizio Popoli and his associates (Musazzi et al., 2010) showed that acute behavioral stress (i.e. unpredictable footshock) induces accumulation of SNARE complexes in PFC neurons. Moreover, recent studies indicate that corticosterone induces an increase in VGLUT2 expression (Lussier et al., 2013). Glucocorticoids may also have a role in the regulation of glutamate transporters. In rats exposed to stress, Autry and colleagues (2006) observed an increase in GLT1b (an EAAT2 isoform) in the hippocampus.

The effects of glucocorticoids on glutamatergic transmission are mediated by both MR and GR. Activation of MR-related membrane receptors enhances excitatory neurotransmission in the hippocampus (Karst et al., 2005; de Kloet, Karst and Joëls, 2008), thereby causing a boost of the initial reaction to stress. Binding of glucocorticoids to MR produces a non-genomic effect by enhancing the probability of glutamate release (de Kloet, Karst and Joëls, 2008). Glucocorticoids also modulate neurotransmission through genomic effects mediated by MR and GR. Specifically in the hippocampus, these two receptors positively modulate glutamatergic transmission (Wang and Wang, 2009; Chatterjee and Sikdar, 2014). Remarkably,

a recent study performed by Nasca and colleagues (2014) demonstrated that stress-induced activation of MR leads to decreased expression of mGluR2, thus reducing an inhibitory constraint on glutamate release in the hippocampus and PFC.

In addition to what is observed presynaptically, exposure to acute stress also regulates postsynaptic AMPA and NMDA receptors in the mPFC (Yuen et al., 2011) and AMPA receptors in the hippocampus (Karst and Joëls, 2005). Glucocorticoids may also affect glutamatergic transmission by altering glutamate receptor trafficking (reviewed by Popoli et al., 2012). These modifications may lie at the core of changes in synaptic plasticity and subsequent behavioral alterations in response to stress.

Nowadays, it is widely accepted that chronic exposure to stressful events may lead to the development of depressive and anxious disorders (Praag, 2004). Changes in the expression and/or effects of glutamate receptors may underlie these disorders. mGluRs might also be involved in stress-related disorders. In particular, modulation of group-I and -II mGluR activity has been shown to be a relevant feature in the treatment of anxiety-related illness. Presynaptic mGluR2 and mGluR3 negatively modulate glutamate release. Thus, agonists for these receptors have been clinically developed for the treatment of anxiety disorders (Schoepp et al., 2003; Swanson and Schoepp, 2003). It has also been demonstrated that mGluR5 negative allosteric modulators decrease anxiety- and depressive-like behaviors in rodents (Tatarczyńska et al., 2001). These effects are mediated by mGluR5 blockade in the hippocampus and amygdala (Riedel et al., 2000; Schulz et al., 2001). Of note, group-III mGluRs share with mGluR2/3 the ability to inhibit both glutamate and GABA release and may therefore be targeted by anxiolytic drugs (Schrader and Tasker, 1997; Semyanov and Kullmann, 2000; Swanson et al., 2005). The mGluR7 in particular appears to be a key regulator of stress reponse. Accordingly, genetic deletion or pharmacological blockade of mGluR7 decreases anxiety-/depressive-like behavior (Cryan et al., 2003; O'Connor et al., 2010; Gee et al., 2014). In contrast, genetic deletion of mGluR8 enhances anxiety (Linden et al., 2002).

Because of their localization in a variety of hypothalamic nuclei as well as in the pituitary gland, mGluRs modulate neuroendocrine functions (reviewed by Durand et al., 2008). Interestingly, glutamate modulates oxytocin release through the activation of AMPA recpetors and group-I mGluRs (Pampillo et al., 2001). The latter are also involved in the regulation of AVP secretion (Morsette, Sidorowicz and Sladek, 2001). Glutamatergic neurotransmission has long been implicated as a critical regulator of the stress response (Van den Pol et al., 1990; Brann, 1995). For example, glutamate stimulates ACTH secretion when injected i.c.v.

(Darlington et al., 1989) or infused into the PVN of the hypothalamus (Makara and Stark, 1975). Also, modulation of mGluR activity has been shown to induce changes in corticosterone levels. While endogenous activation of group-I and -III mGluRs increases corticosterone secretion, group-II mGluR instead exert a tonic control on mild stress (Durand et al., 2008).

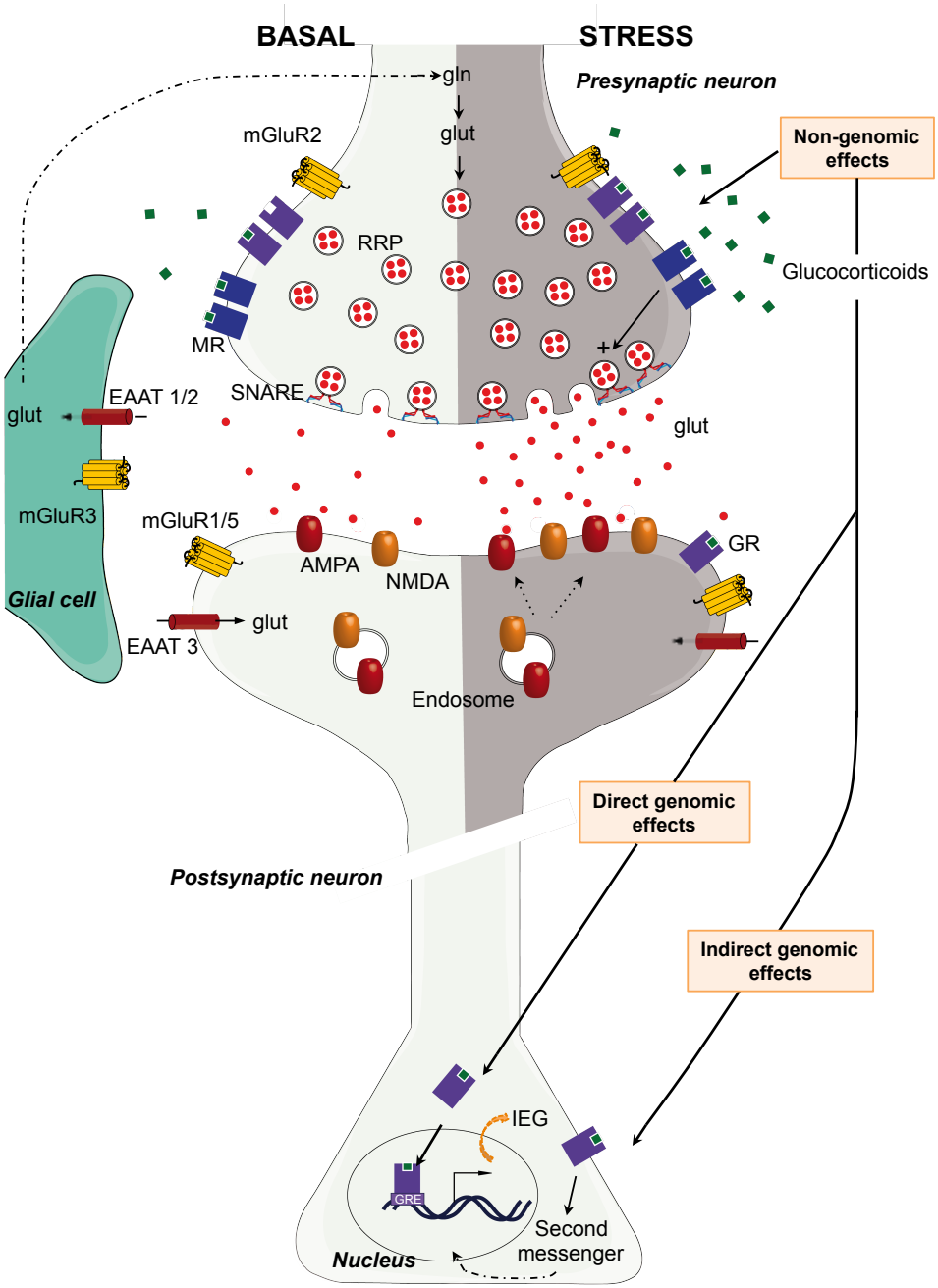

Figure 8: Effects of glucocorticoids on the neurotransmission of the tripartite glutamate synapse.

Neuronal glutamate (glut) is synthesized *de novo* from glucose and glutamine (gln) supplied by glial cells. Glutamate is then packed in synaptic vesicles that will be mobilizable upon stimulation, i.e. the readily releasable pool (RRP). Vesicles will then fusion with the presynaptic membrane thanks to SNARE complex proteins. After release into the extracellular space, glutamate binds to ionotropic glutamate receptors (NMDA and AMPA) and metabotropic glutamate receptors (mGluR) on the membranes of both postsynaptic and presynaptic neurons as well as glial cells. Glutamate is cleared from the synapse through excitatory amino acid transporters (EAAT) on neighboring glial cells (EAAT1 and -2) and, to a lesser extent, on neurons (EAAT3and -4). Glucocorticoids exert their action through (*i*) non-genomic effects mediated by the activation of mineralocorticoid receptors (MR) that stimulate glut release; (*ii*) indirect genomic mechanisms mediated by membrane receptor and second messengers, mainly glucocorticoid receptors (GR), and (*iii*) genomic actions mediated by cytoplasmic receptors (GR) that move to the nucleus and act as transcription factor to activate transcription of stress-related genes as well as immediate early genes (IEG).

1.2.3. Stress and regulation of memory processes

The hippocampus plays a crucial role in stress-induced processes related to memory, particularly spatial and contextual learning and memory retrieval (Roozendaal and McGaugh, 1997). Depending on the time of exposure and the duration and type of stressor, stress has divergent effects on learning and memory (Diamond et al., 2007; Lupien et al., 2009). As discussed above, acute stress is tightly associated with an increase in excitatory transmission with an ensuing improvement in working memory processes both in rodents (Yuen et al., 2009; 2011) and humans (Lupien et al., 2002; Smeets et al., 2006). In addition, exposure to acute stressful events increases associative learning (Beylin and Shors, 2003; Conboy and Sandi, 2010). Stress also facilitates classical conditioning (Shors, Weiss and Thompson, 1992). Indeed, this phenomenon is part of the adaptive response to stress. On the contrary, chronic stress (or long-lasting glucocorticoid treatment) impairs PFC-dependent cognitive function in rodents (Cerqueira et al., 2005) and humans (Young et al., 1999; Liston et al., 2009), as well as hippocampus-dependent cognitive processes (McEwen, 2001; 2007). Accordingly, Kim and colleagues (2007) showed that stress alters the firing properties of place cells in the hippocampus, which are thought to support spatial navigation and memory (O'Keefe and Dostrovsky, 1971). Very recently, Tomar and colleagues (2015) demonstrated the existence an adaptive process of CA1 place cells, the firing of which is decreased after 5 days of repeated stress but is no longer evident after 10 days.

Limbic structures, and particularly the hippocampus, are highly sensitive to stress, and exposure to adverse events associated with high levels of glucocorticoids impairs performance on memory tasks dependent on the hippocampus (McEwen and Sapolsky, 1995; Kim and Diamond, 2002). In human studies, evidence that high levels of glucocorticoids are deleterious for mnemonic processes is found in patients with hypercortisolism (Starkman et al., 1992). Mounting evidence shows that chronic stress induces alterations in the structure of glutamatergic synapses such as atrophy, dendritic retraction and/or spine loss (reviewed by Popoli et al., 2012). In the adulthood, the limbic system still presents high levels of plasticity that involves synapses strength as well as renewal of cells destined to be integrated in pre-existing neuronal network.

a) Effects of glucocorticoids on synaptic plasticity

Activity-dependent synaptic plasticity underlies associative learning (Hebb, 2005). Hence, stress-induced changes in learning and memory mainly involve modifications in synaptic strength (Howland and Wang, 2008). The two most studied models of activity-dependent

synaptic plasticity are LTP and LTD in the hippocampus (Bliss and Collingridge, 1993; Cooke and Bliss, 2006). Both LTP and LTD are sensitive to stress. In 1987, Foy and colleagues found an impaired LTP in hippocampal slices prepared from stressed rats. However, the effect of stress on LTP is controversial and depends on the type of stress, the area of investigation and the electrophysiological paradigm of LTP induction (reviewed by Kim, Pellman and Kim, 2015). There is evidence that selective activation of MR increases LTP, whereas recruitment of GR decreases LTP and enhances LTD (Pavlides et al., 1995). These findings suggest that low levels of glucocorticoids enhance LTP through preferential stimulation of the high-affinity MR, whereas during stress, increasing levels of glucocorticoids activate low-affinity GR, leading to LTP impairment (McEwen and Sapolsky, 1995). Accordingly, pharmacological blockade of GR is able to prevent stress-induced impairment of LTP in the hippocampus and frontal cortex (Maillet et al., 2008).

b) Glucocorticoids and regulation of neurogenesis

Glucocorticoids regulate hippocampal neurogenesis, which has been implicated in memory processes. Cameron and Gould (1994) demonstrated that acute corticosterone administration enhances neurogenesis in the hippocampal DG, whereas adrenalectomy produced the opposite effect. Adult hippocampal neurogenesis has also been demonstrated in humans (Gould and Gross, 2002). In the hippocampus, neurogenesis may provide a cellular support for the increasing demand associated with learning and memory. Indeed, new learning is associated with increasing number and survival of new neurons (Nottebohm, 2002; reviewed by Deng, Aimone and Gage, 2010). Conversely, deficits in hippocampus-related tasks are correlated with decreasing number of new DG granular cells (Shors et al., 2000).

Exposure to stressful events has deleterious effects on hippocampal neurogenesis. Increased levels of glucocorticoids as well as increased excitatory neurotransmission have suppressive effects on neurogenesis (Gould et al., 1997). It has also been shown that chronic stress in rodents leads to anatomical modifications in the hippocampus by reducing adult neurogenesis in the DG (Pham et al., 2003). This evidence suggests that formation of new granular cells might also contribute to the modulatory action of the hippocampus on the HPA axis (Herman et al., 1989). Remarkably, despite the major role of glucocorticoids in this cellular process, other circulating hormones like oxytocin also regulate neurogenesis under stress conditions. In rodents exposed to stress or treated with glucocorticoids oxytocin was able to stimulate adult neurogenesis (Leuner et al., 2012).

The analysis of patients with Cushing's syndrome has confirmed the deleterious effects of glucocorticoids on the CNS. Patients with hypercortisolemia show defects in learning and memory associated with a reduced hippocampal volume (Starkman et al., 1992). These abnormalities are corrected by treatments that normalize glucocorticoid levels (Starkman et al., 1999). Stress-related disorders, major depression in particular, are associated with atrophy of specific brain regions, including the hippocampus (Sapolsky, 2000; Sandi, 2004; Lupien et al., 2009). However, volumetric MRI analysis in rodents failed to demonstrate a reduction in hippocampal volume in response to chronic restraint stress (Lee et al., 2009). When present, changes in hippocampal volume might be in part accounted by a reduced neurogenesis caused by a rise in glucocorticoid levels, as physiologically occurs during ageing (Sapolsky, 1992; Seki and Arai, 1995; Kuhn, Dickinson-Anson and Gage, 1996; Cameron and McKay, 1999). While in the short term adrenal steroid have the ability to enhance synaptic function and promote adaptive modifications, a long-lasting rise in glucocorticoid levels may cause irreversible damage of the hippocampus. In line with this view, acute activation of the HPA axis during learning can facilitate memory consolidation (Roozendaal et al., 1999; Sandi, 2004), whereas chronic exposure to stress may predispose to to cognitive deficits in later life (Lupien et al., 2009; Sandi, 2004).

1.2.4. Glucocorticoids contribute to brain aging

Aubrey de Grey (1999) defined aging as "deteriorative changes with time during post-maturational life that underlies an increasing vulnerability to challenges, thereby decreasing the ability of the organism to survive". Over decades, different theories explaining the ageing phenomenon and associated physiological changes have been suggested (Table 2). Of note, the effect of aging among individuals is highly variable, "pathological aging" (a poorly defined definition) is a major health concern because of the growing number of oldest age segments of the population.

Table 2: Major theories explaining aging processes
(adapted from Weinert and Timiras 2003).

Theories of ageing
Evolutionary theory: Mutated and normal genes, which are not harmful at younger age, could be deteriorating to late life.
Molecular theory: Accumulation of somatic mutation, changes in gene expression, increase of proteins abnormalities due to lack of efficiency of protein-synthetic apparatus, and impairment of translation process cause aging.
Neuroendocrine control theory: Alterations in neuroendocrine control of homeostasis results in age-related physiological changes.
Free-radical theory: Aging occurs as a cost of cellular damages such as lipid (membrane), protein, and DNA damage due to high reactive free radicals that are produced during oxidative metabolism.
Telomere theory: Cells reach a senescent state associated with loss of telomere during DNA replication.

Exposure to stress is one of the factors explaining the variability of variable age-related changes in brain function. Individual differences in HPA activation in humans and associated differences in glucocorticoids secretions lie at the core of variation in susceptibility to glucocorticoid-accelerated aging (Goosens and Sapolsky, 2007). Life-long exposure to glucocorticoids may be responsible for damage to sensitive neurons (particularly to hippocampal glucocorticoid-sensitive neurons), which in turn reduces their inhibitory control of glucocorticoids on the HPA axis. As outlined above, glucocorticoids levels gradually increase over time (Sapolsky, Krey and McEwen, 1984; 1986). This feed-forward effect on hippocampal function is known as the "glucocorticoid cascade hypothesis" and is proposed to contribute to reduction in the number of hippocampus neurons and cognitive impairment observed in some aged individuals (Sapolsky, Krey and McEwen, 1985).

Several lines of evidence suggest a role for glucocorticoids in age-related cognitive decline. In 20 month-old Fisher male rats, basal levels of ACTH and corticosterone were found to be higher than in 3 month-old rats (Hauger, Thrivikraman and Plotsky, 1994), and inhibition of ACTH secretion by the glucocorticoid, dexamethasone, was blunted middle-aged Sprague-Dawley rats as compared to young rats (Revskoy and Redei, 2000). The age-related increase in stress hormones is associated with cognitive decline. Dellu and colleagues (1994) have suggested a relationship between mnesic deficits and increased corticosterone secretion. Along this line, adrenalectomized mid-aged rats maintained with chronic low levels of glucocorticoids showed a reduced cognitive decline (Landfield, Baskin and Pitler, 1981).

Brain regions that are tightly involved in cognitive functions undergo important functional modifications across life, and exposure to stressful event as well as physiological elevation of glucocorticoids levels during aging might contribute to these changes (Lupien et al., 1998; Sapolsky, 1999; Miller and O'Callaghan, 2003), leading sometimes to the development of pathological states. The aged hippocampus consistently shows anatomical, neurochemical, and electrophysiological alterations that might contribute to cognitive decline (Miller and O'Callaghan, 2003). Glucocorticoids may have a role in these changes, as suggested by the high levels of glucocorticoid receptors in the hippocampus (Porter and Landfield, 1998). Whereas some types of mild stress (e.g., caloric restriction) might be beneficial and reduce the risk of mental and systemic disorders associated with aging, some types of psychosocial stress may have detrimental consequences (reviewed by Pedersen, Wan and Mattson, 2001). Studies in non-human primates have shown that high levels of glucocorticoids cause long-term neuronal damage in the CA3/CA2 subregions, such as dendritic atrophy and cell body shrinkage (Sapolsky et al., 1990).

Interestingly, glucocorticoid secretion is increased in age-related human disorders associated with cognitive dysfunction, such as Alzheimer's disease (AD; Armanini et al., 2003; Dai, Buijs and Swaab, 2004). As highlighted above, the HPA axis is chronically activated at least in some types of depression (O'Brien et al., 2004; de Kloet, Joëls and Holsboer, 2005), and depression is one of the early psychiatric manifestations of AD. Psychiatrists now place more and more emphasis on cognitive dysfunction associated with stress-related disorders. For example, a large proportion of depressed patients show cognitive symptoms (such as defects in attention, cognitive flexibility, executive function, working memory, and episodic memory) that are dissociated from the severity of the hallmark features of depression, such as decreased mood and anhedonia. These changes should not be confused with the difficulty to think or concentrate often reported by depressed patients or with the so-called "hot cognition", i.e., the propensity of depressed patients to process external stimuli with a "negative" valence at the cost of stimuli with a "positive" valence (reviewed by Goeldner et al., 2013). Stress-related changes in the physiology of the hippocampus and other glucocorticoid target regions may lie at the border of mood disorders and cognitive dysfunction associated with physiological and pathological aging.

1.3. Stress and metabolic control

1.3.1. Stress: the "sweet enhancer", mobilization of energy resources

Hormonal pathways activated in response to stress cause a reorganization of energy balance. As the name "gluco-corticoid" indicates, primary role of these steroids is to mobilize sugar resources (McMahon, Gerich and Rizza, 1988; Dallman et al., 1993) and consequently enhance capacity to respond to an ongoing or anticipated challenge and incite "fight-or-flight" responses. Coping with stress involves the concerted activity of multiple interacting central stress-regulatory systems to mobilize energy for the organism (Myers, McKlveen and Herman, 2014). Since the beginning of the 20th century, Walter Cannon (1929) found anticipatory increase in glucose levels in college rowers the night before a competition.

Glucocorticoids enhance blood glucose levels by increasing hepatic glucose production and decreasing peripheral glucose uptake by skeletal muscle and fat cells (Dinnen et al., 1993). Glucocorticoids are members of a large group of counter-regulatory hormones that include circulating catecholamines, glucagon, growth hormone, angiotensin II, and β-endorphin, *inter alia*. Administration of cortisol in high concentrations, which mimic those present during the early morning peak in humans, increases both fasting and postprandial glucose concentrations (Dinnen et al., 1993). This scenario is reminiscent of that encountered in type-2 diabetes, a disorder characterized by insulin resistance and hyperglycemia (Yuen, McDaniel and Riddle, 2012). Accordingly, individuals with high cortisol levels exhibit elevated glucose and triglyceride levels as well as decreased insulin sensitivity (Phillips et al., 1998), and so do mice chronically treated with corticosterone (Karatsoreos et al., 2010). All these are phenotypical allmarks of the so-called "metabolic syndrome", which represents a major risk factor for cardiovascular disorders and is in comorbidity with stress-related psychiatric disorders, such as major depression. Of note, exposure to chronic stress is may lead to the development of metabolic syndrome (Gohil et al., 2001).

Glucocorticoid-induced hyperglycemia may in principle up-hold energy provision in CNS neurons, which mainly use glucose (and not free fatty acids) as substrate for oxidative metabolism and ATP production. This mechanism may be aimed at reinforcing learning and memory processes associated with stress (Osborne et al., 2015). It is generally believed that glucose can cross the blood-brain barrier without the aid of insulin, which is instead necessary for glucose uptake in skeletal muscle and fat tissue. However, activation of insulin/insulin-like growth factor family of membrane receptors is necessary for glucose uptake in neurons (see

below). In addition, high levels of glucocorticoids may reduce oxidative glucose metabolism in neurons as they do in hepatocytes, thereby contributing to chronic-stress induced hippocampal damage (Goodman et al., 1996). There is also a tight relation between glucose homeostasis and neurotransmission. Hence, regulation of glucose metabolism has a high impact on cognition and mental health in general (Hendrickx, McEwen and Ouderaa, 2005). Gold and colleagues (2007) found that type-2 diabetes was associated with reduced hippocampal volume and reduced cognitive performances.

1.3.2. Glucose homeostasis: emerging role for oxytocin

Maintenance of glucose homeostasis is essential to ensure adequate nutrient flow to all tissues. Because glucose is the main fuel of the CNS, this regulation is particularly important in the brain, which integrates all nutritional information and also actively regulates energy balance (reviewed by Grayson, Seeley and Sandoval, 2013). The brain stress-response system and the one controlling energy homeostasis share neuroanatomical substrates that mainly converge in the hypothalamus. The latter is the conductor of the neuroendocrine orchestra and regulates whole body energy homeostasis (Myers and Olson, 2012). Indeed, regulation of energy balance and brain homeostasis in general is the result of a concerted action of antagonist endocrine systems. In particular, the PVN of the hypothalamus is the crossroads for both metabolic and stress-related glucocorticoids actions. One peptide originating from parvocellular cells of the PVN has gain attention in the context of metabolic regulation. Mainly known for its role during labor and early *postpartum* period, there are mounting indications that oxytocin physiological effects are significant even away from pregnancy exerting various physiological actions. Besides its active role as an “anti-stress” hormone (described above), oxytocin has emerged as an important anorexigen in the regulation of energy balance (Ho and Blevins, 2013). This role in reducing food intake was first demonstrated in rodents with peripheral and central administration of oxytocin (Arletti, Benelli and Bertolini, 1989). Ever since, different studies have associated alterations in oxytocin signaling with changes in energy balance. Oxytocin is released in a pulsatile fashion, and peak circulating levels generally have recently been shown to correspond with typical patterns of food intake in mice (Zhang and Cai, 2011). Likewise, hypothalamic oxytocin mRNA expression is reduced in fasting and restored upon food intake (Kublaoui et al., 2008) and plasma levels of oxytocin rise with food intake (Verbalis et al., 1986).

Oxytocin also contributes to regulate glucose homeostasis. Indeed, release of oxytocin has been associated to insulin-induced hypoglycaemia in healthy individuals (Lippert et al., 2003).

Hypoglycaemic effect of oxytocin might be mediated by peripheral increase in glucose uptake *via* insulin-like signaling pathway (Lee et al., 2008; Florian et al., 2010). Interestingly, this hypoglycaemic effect was significantly higher in diabetic patients (Volpi et al., 2013). Besides, animal studies have highlighted that oxytocin is involved in carbohydrate metabolism. Infusion of oxytocin induces a rise in plasma glucose, insulin and glucagon levels (Altszuler and Hampshire, 1981; Knudtzon, 1983; Dunning, Moltz and Fawcett, 1984). This effect was associated to a direct action on the pancreas (Stock et al., 1990). In rodents, further investigations highlighted that oxytocin induced an enhanced glucagon secretion in control rats and to a much greater extent in streptozotocin-induced diabetic conditions (Widmaier, Shah and Lee, 1991). Mice lacking oxytocin exhibit decreased insulin sensitivity and impaired glucose tolerance (Camerino, 2009), whereas daily injections of peripheral oxytocin improved glucose tolerance even in mice maintained on high-fat diet (Zhang and Cai, 2011). In the last years, since oxytocinergic system affects glucose and insulin homeostasis, it has bee suggested as a possible target for the treatment of obesity and diabetes (Deblon et al., 2011; Zhang et al., 2013; Elabd, Mohasseb and Algendy, 2014). Beneficial role of oxytocin passes through activation of intracellular cascades *via* binding to the oxytocin receptor as described in figure 9. Of note, mice deficient in the oxytocin receptor gene develop impairment in energy balance regulation revealed by an obese phenotype and aberrations in body temperature (Nishimori et al., 2008).

Regulation of energy balance as well as glucose homeostasis exerted by oxytocin involves relevant sites of action in both brain and periphery. Within CNS, areas containing OTR include the ventromedial nucleus of the hypothalamus, the amygdala, the lateral septum, the BNST, the preoptic and ventral tegmental area as well as the hippocampus (reviewed by Viero et al., 2010). Peripherally, a plethora of organs distinct from the usual endocrine systems where oxytocin plays biological roles express OTR (e.g., reproductive system, heart, pancreas). Since oxytocin acts in a positive feedback loop manner to stimulate its own release it is complicated to decipher whether its action on energy modulation is more central or peripheral. It is likely that oxytocin beneficial role result from a complex interaction with other endocrine systems (e.g., glucocorticoids, insulin). This is particularly relevant in the framework of stress. Indeed, there is a tight association between stressful conditions and variation in individual weight as well as obesity (Dallman, 2010) and oxytocin neurons are activated in both stressful conditions and food intake (Onaka, Takayanagi and Yoshida, 2012). Hence, the oxytocinergic system is an important feature in the study of stress-related and metabolic disorders.

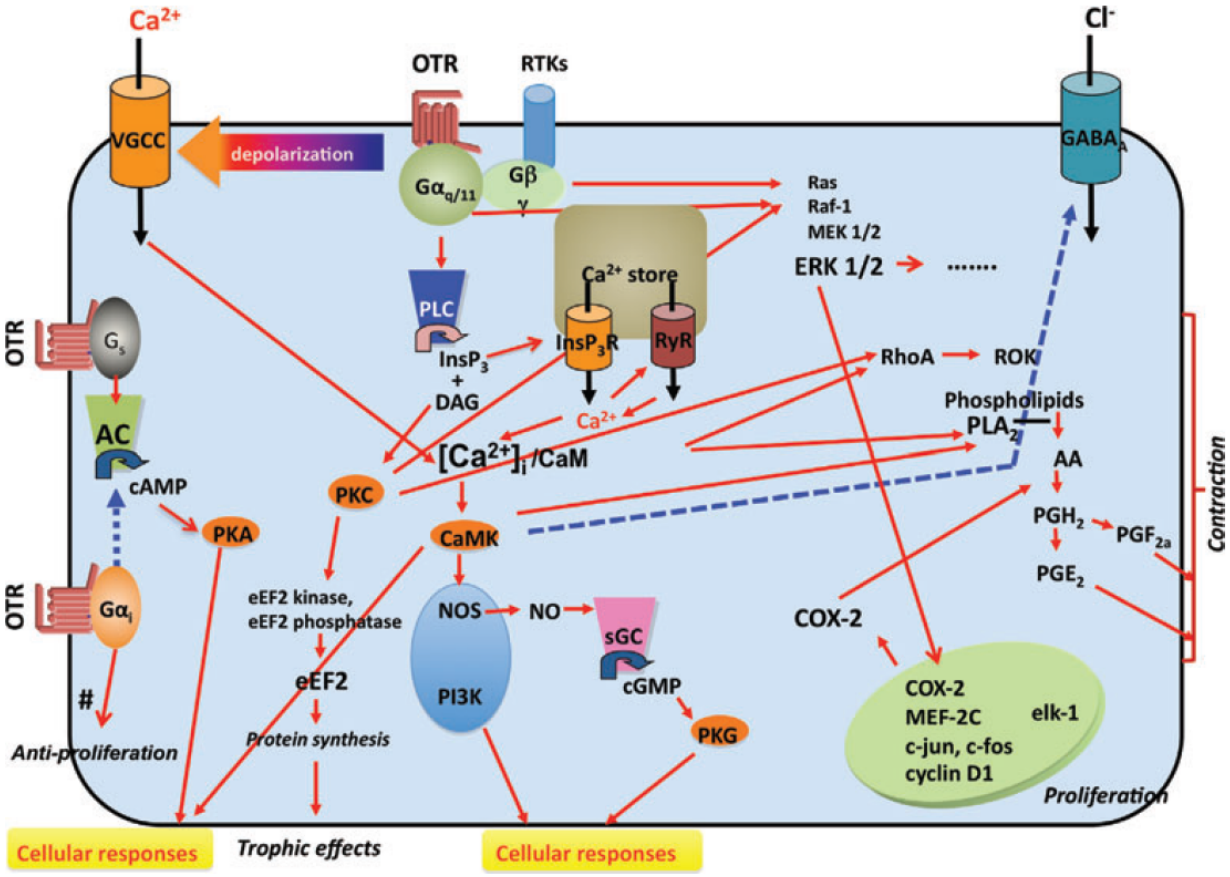

Figure 9: Schematic diagram of OTR-linked signaling pathways.

Oxytocin receptor (OTR) activation leads to three different GTP-binding protein mechanisms. The major mechanism is mediated by the Gq/PLC/InsP3 pathway. When oxytocin binds to OTR, it activates Gαq/11 and then phospholipase C (PLC), which induces the cleavage of PIP2 to inositol trisphosphate (InsP3) and diacylglycerol (DAG). InsP3 induces Ca^{2+} release from Ca^{2+} stores via InsP3 receptor (InsP3R) and, in some cells, causes Ca^{2+}-induced Ca^{2+} release (CICR) via the ryanodine receptor (RyR). The activation of Gq also causes membrane depolarization, which, in turn, activates voltage-gated Ca^{2+} channels (VGCC) and then facilitates Ca^{2+} entry through VGCC. Thus, increased cytosolic Ca^{2+} ($[Ca^{2+}]_i$) stimulates Ca^{2+}/Calmodulin-dependent protein kinase (CaMK) after binding to the Ca^{2+} binding protein Calmodulin. The Ca^{2+}-calmodulin complex (Ca^{2+}/CaM) complex then activates CaMK and causes various cellular responses, such as smooth muscle contractions, or induces the activation of several different types of enzymes, such as NOsynthase (NOS) or phosphoinositide 3-kinase (PI3K). DAG causes protein kinase C (PKC) activation and also various cellular responses. Additional pathways activated through the OTR include the MAP-kinase (MAPK) and the Rho kinase (ROK) pathways. The increased transcription of cyclooxygenase 2 (COX2) mediates the increased production and secretion of prostaglandins. The OTR-mediated opening of Ca^{2+} channels is likely mediated through free $G_{\beta\gamma}$ subunits. The OTR is known to be coupled with the other G proteins, Gs and Gi, both of which are linked with the adenylate cyclase (AC) pathway. The proliferative effects involve MAPK-mediated activation of specific gene transcription. The trophic effects are mediated via a PKC-mediated activation of eEF2. Activation of the Rho and MAPK pathways, the increase in intracellular $Ca^{2+}$and the increased prostaglandin secretion all contribute to the contractile effects. The antiproliferative effects observed in certain cells types appear to be mediated via αi G protein subunits. The solid red lines and broken blue lines indicate activation and inhibition, respectively (from Viero et al., 2010).

1.3.3. Metabolic programming

Regulation of glucose metabolism is a major determinant in late pregnancy. Labor begins when the mother can no longer provide sufficient nourishment to the fetus (Swaab, 2014). Maternal glucocorticoids also play an important role in mechanisms that increase the availability of glucose for the developing fetus by mobilizing substrates for hepatic gluconeogenesis (Reynolds and Walker, 2003). Prenatal challenges, such as nutritional defects and/or exposure to glucocorticoids may cause the development of a pathological programming leading to a variety of disorders in the adult life (Seckl, 2004; Gluckman et al., 2005).

In the last decades, numerous epidemiological studies have shown that early life environment critically influence the occurrence of a variety of disorders in the late life (Fernandez-Twinn and Ozanne, 2010). From the moment of conception, the placenta plays a key role in mediating the amount and quality of nourishment provided to the fetus. Fetal growth depends on an adequate placental nutrient transfer. A primary nutrient for the developing fetus is glucose, which is transported across the placenta down a maternal-fetal concentration gradient (Knipp, Audus and Soares, 1999). Three high-affinity isoforms of glucose transporters are expressed in the placenta: glucose transporters type-1, -3, and 4 (GLUT1, -3, and -4) (Korgun et al., 2001). GLUT1 mediates glucose transport from the maternal blood to the placenta and from the placenta to the fetus (Zhou and Bondy, 1993), whereas expression GLUT3 is restricted to cells on the fetal surface of the placental barrier (Boileau et al., 1995). GLUT4 expression is very low in rodent placentas but highly expressed in humans (Xing et al., 1998). Any factor that perturbs the fetal-maternal interface may cause intrauterine growth restriction and defective organ formation, and may predispose to major health consequences by hindering developmental trajectories (Edwards et al., 1993; Seckl, 2001). Indeed, Mairesse and coworkers (2007) showed that maternal stress in rats affect placental nutrient transfer capacity by modifying GLUTs expression in the placenta of stressed dams. Also, chronic maternal stress affects growth and organ development of the fetus, resulting in a decrease body weight at birth (Mairesse et al., 2007a).

In conclusion, a mother may transmit a signal to the fetus ‘it is tough out here’ because of reduced food availability and/or increased stress. This will cause biochemical changes in the fetus and affect the offspring in order to tip the balance in favor of survival and insure possibility to reproduce. However, in the post-reproductive period these changes may be disadvantageous. This particularly occurs if the adult environment does not match that for which developmental plasticity is aimed. Thus, if early-life undernutrition programs a ‘thrifty

phenotype' and then food is plentiful, the adult may be at risk of developing metabolic diseases such as diabetes and obesity (Gluckman and Hanson, 2004).

2. Perinatal stress and early programming of neurological disorders

As Richard Wollheim (1923 – 2003) said, "a good environment is not a luxury, it is a necessity". The individual ability to cope with stressors is the result of a complex interaction between nature and nurture. Understanding how nature (gene) and nurture (environmental stimuli) influence each other is critical to explain individual variations in the vulnerability to the development of stress-related disorders. Vulnerability to develop chronic diseases may be programmed early in life, and, in particular, during the fetal period, which is critical for shaping the lifelong health of an individual (Barker, 1995). This led to the elaboration of the theory of the developmental origins of health and disease (DOHaD).

Early exposure to environmental stressors may have a long-lasting effect on the developing organism, thereby programming or "imprinting" persistent changes in fetal structure, physiology and metabolism. Preclinical and clinical studies have shown that the brain is particularly sensitive to stress during infancy. Interestingly, the same occurs during aging. Thus, infancy and aging are considered as "critical periods" for the brain response to stress (Lupien et al., 2009; Fig. 10). Early stressful events may produce either adaptive or maladaptive consequences in the offspring (Gluckman and Hanson, 2004), which are initially aimed at supporting survival, but then predispose the individual to later life disease, particularly if the environment diverges from that predicted by early life conditions. If so, early life events may cause the late development of several neurological, metabolic and neuroendocrine abnormalities (Seckl, 1998; Lupien et al., 2009) that can persist all life long. Since an important feature of the stress response is the secretion of high levels of glucocorticoids, these steroids have become an obvious mediator and target for "(re)programming action" in early life stress paradigms (Painter, Roseboom and de Rooij, 2012; Maccari et al., 2014). During the prenatal period, glucocorticoids play a major role in fetal development, including the development of the HPA axis.

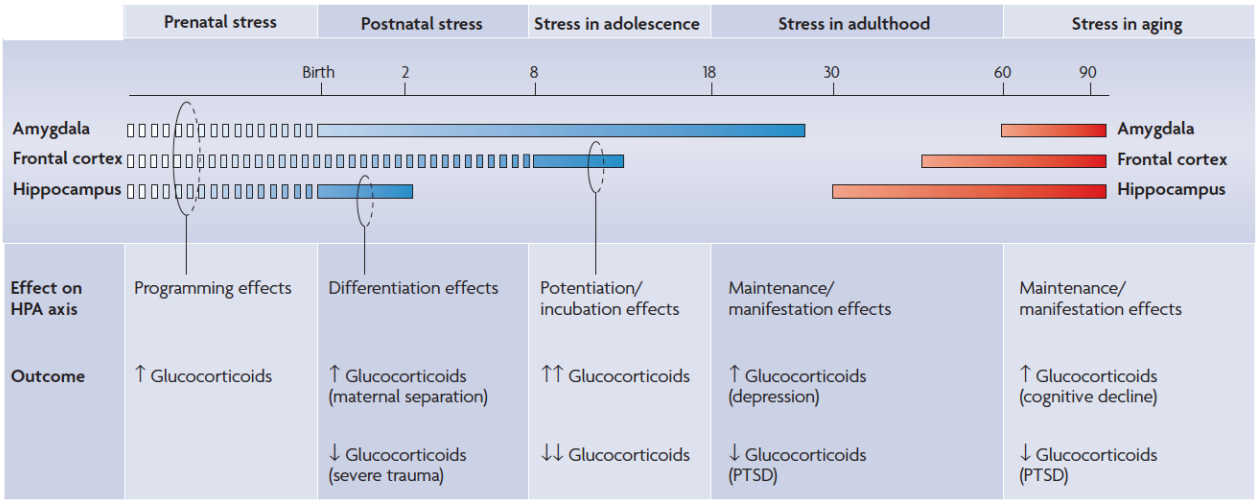

Figure 10: The life cycle model of stress.

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

2.1. Ontogeny of the hypothalamic-pituitary-adrenal axis and models of hormonal programming

2.1.1. Glucocorticoids, an important trigger for fetal development

Glucocorticoids are an important developmental trigger in most mammalian species and they specifically affect systems involved in fetal growth *via* the transcriptional activity of MRs and GRs. The HPA axis of the mother undergoes major changes during pregnancy (Lindsay and Nieman, 2005). During late gestation, there is a surge of glucocorticoid levels, which serves to promote fetal lung maturation, *via* stimulation of pulmonary surfactant production (Ward, 1994). This is partly due to the stimulation of maternal pituitary and adrenal glands by placental CRH (reviewed by Reynolds, 2013; Fig. 11) with a resulting increase in cortisol secretion. In addition, glucocorticoids are critical for developmental switching in many other organ systems essential for life after birth, including thyroid, kidney, brain and pituitary (Fowden, Li and Forhead, 1998). Of note, *in utero* ablation of the PVN of the fetal hypothalamus, pituitary and adrenal gland results into a prolonged gestation, whereas fetal infusion of ACTH or glucocorticoids during late gestation is a precipitating factor for premature delivery (reviewed by Challis et al., 2001). Increases in glucocorticoids levels during the whole gestation finally triggers parturition.

Interestingly, glucocorticoids promote a correct brain development by triggering terminal maturation of neurons and axon/dendrite remodelling, and also affect cell survival (Meyer, 1983; Yehuda, Fairman and Meyer, 1989). It appears that the time-window during which glucocorticoids prime organs maturation must be tightly regulated. Accordingly, exposure of the developing fetus or neonate to excessive or out-of-phase glucocorticoid signals might lead to substantial alterations in normal developmental trajectories, resulting into an altered physiological function throughout life, and eventually pathology (Moisiadis and Matthews, 2014). This is particularly relevant for the CNS and the subsequent development of HPA axis, with major consequences in the stress response later in life (Matthews, 2000).

The fetus is exposed to glucocorticoids produced by the mother or by its own adrenal glands. Maternal glucocorticoids cross the placenta, a tissue that expresses high levels of 11β-HSD2. This enzyme inactivates glucocorticoids and plays an important role in shielding the developing fetus from inappropriately high maternal glucocorticoid levels during development (Edwards et al., 1993; Meaney, Szyf and Seckl, 2007; Fig. 11). However, this placental barrier is apparently incomplete, with 10–20% of maternal glucocorticoids reaching the fetus (Benediktsson et al., 1997).

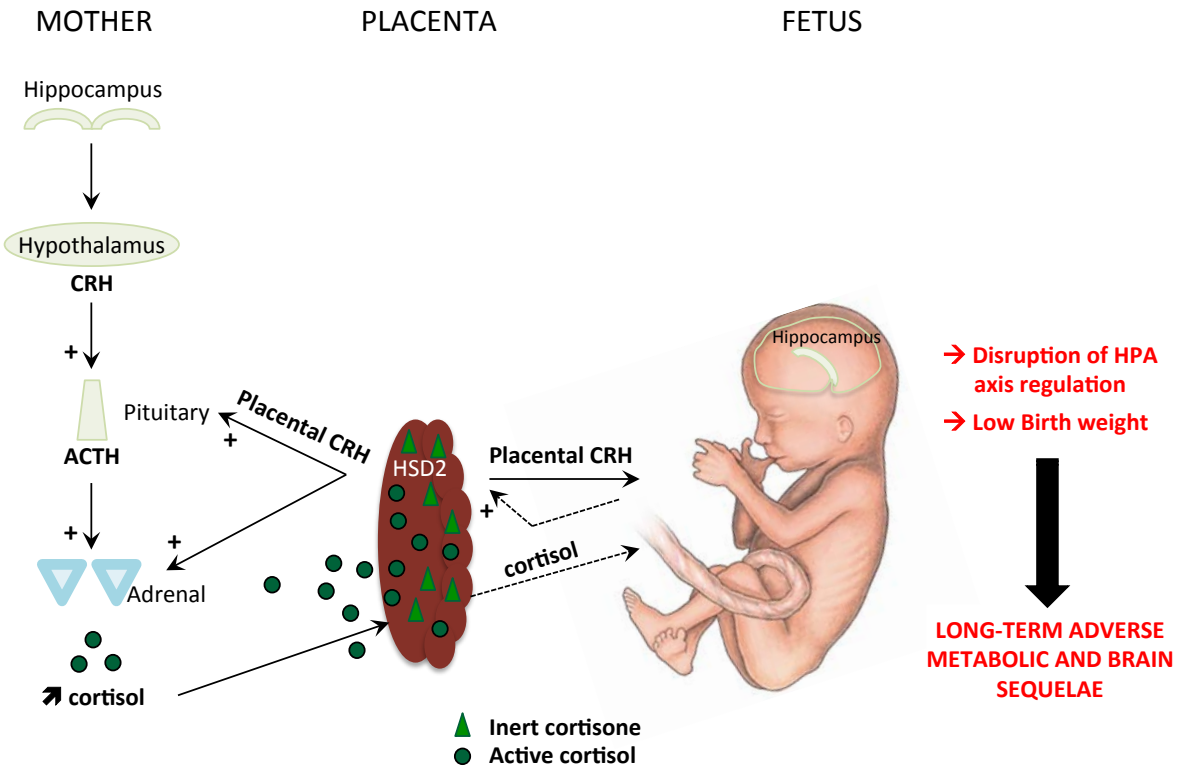

Figure 11: Glucocorticoid signaling between mother, placenta and fetus.
Figure shows interaction between maternal, placental and fetal compartments during pregnancy leading to overexposure of the developing fetus to glucocorticoids. Activation of the maternal HPA axis during pregnancy leads to increased circulating levels of cortisol (filled circles). Placental CRH also directly stimulates the maternal pituitary and adrenal to further increase cortisol levels, while maternal cortisol also stimulates placental CRH production. Maternal cortisol passes through the placenta where it is broken down by the enzyme HSD2 into inactive cortisone (green triangles). The fetus can also signal to the placenta to increase production of placental CRH when fetal metabolic demands increase. Overexposure of the developing fetus to excess cortisol leads to fetal HPA axis activation which is associated with low birthweight and long term adverse programmed outcomes including metabolic and brain sequelae. CRH — corticotropin releasing hormone, ACTH — adrenocorticotropin hormone, HSD2 — 11β hydroxysteroid dehydrogenase type 2 (adapted from Seckl, 2001; Erhuma, 2012; Reynolds, 2013).

In spite of the physiological importance of glucocorticoids for fetus development, stress-induced increases in glucocorticoid levels during gestation are detrimental. Initial evidence that exposures to adversity early in life could profoundly modify the responsiveness of the HPA axis to stress later in life was provided in the 50's (Levine, 1957). Since then, studies carried out in various animal species have demonstrated that neurological, endocrine, metabolic and cardiovascular function and dysfunction, but also psychiatric disorders, found in adulthood have developmental origins (Harris and Seckl, 2011; Santos and Joles, 2012; Entringer and Wadhwa, 2013). Endogenous levels of glucocorticoids in the mother and fetus might be altered

under some specific circumstances as maternal adversity (for example, hypo- or malnutrition), anxiety, depression, or fetal stress (as occurs in response to hypoxia). As such, glucocorticoid programming of the HPA axis has been identified as a pivotal link between early life experience and the development of chronic disease later in life (Reynolds, 2013). Maternal glucocorticoids program the fetus to stress-related disorders by inducing permanent changes in the regulation of the HPA axis and in the functioning of brain regions that are critically involved in the response to stress, such as the hippocampus (Seckl and Meaney, 2004).

2.1.2. Deciphering the perinatal programming: interests and validity of animal models

Investigation of mechanisms that lie at the core of the perinatal programming requires the development of appropriate tools. In neurobiology, there are ethical and practical difficulties of examining the living human brain. Thus, specific animal models have been developed in order to decipher intrinsic actors of human neurobehavioral disorders and discover possible new candidates for therapeutic intervention. Nevertheless, judgment criteria are needed for evaluation of the legitimacy of a given model, especially from a translational standpoint (Nestler and Hyman, 2010). A multidimensional set of validity criteria was elaborated since the early 1960's but these criteria were originally applied to screening tests rather than to the choice of animal models (for review see Belzung and Lemoine, 2011). After almost twenty years Willner suggested three criteria of "validities" that could be particularly applied to animal models for psychiatric disorders (Fig. 12): *i)* the "construct" or etiologic validity, which refers to the disease relevance of the methods by which a model is constructed. In the ideal situation, construct validity is achieved by recreating in animals the etiologic processes that cause a human disease, thus replicating the neural and behavioral features of the illness. This can be achieved by genetic manipulations (e.g., knock-out or transgenic mice) or by exposing animals to well-validated environmental risk factors or known disease-causing agents (e.g., stress as in the present study); *ii)* "face" validity indicating that a particular animal model recapitulates important anatomical, biochemical, neuropathological or behavioral features of a human disease; and, *iii)* "predictive" or pharmacological validity indicating that a model responds to treatments in a way that predicts the effects of those treatments in humans. No perfect animal model exists for CNS disorders in general and psychiatric disorders in particular. However, the choice of appropriate animal models as is a *condition-sine-qua-non* for translational research that paves the way to new treatments for human disorders (Markou et al., 2008).

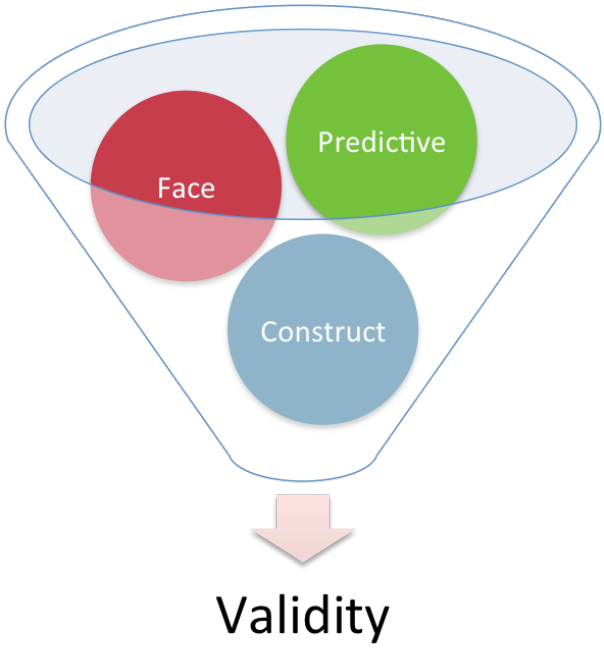

Figure 12: Set of animal models' validity criteria

2.1.3. Animal models of early-life stress: different early life exposures but common long-term outcomes on offspring health

In humans, there is a clear association between low birth weight and the development of hypertension, type-2 diabetes and cardiovascular disease in adulthood (Barker et al., 1993; Fall et al., 1995). Low body weight is also associated with psychiatric disorders such as schizophrenia, attention deficit/hyperactivity disorder (ADHD), antisocial behavior, increased vulnerability to post-traumatic stress disorder (PTSD), anxiety disorders, learning difficulties and depression (reviewed by Harris and Seckl, 2011). If low body weight *per se* cannot be the cause of such disorders, it appears to be a good, even though unsophisticated, indicator of fetal programming. Experience of reduced birth at weight is often followed by rapid postnatal catch-up growth as well as altered fat content and distribution, which may predispose to cardiometabolic disorders (Owen and Matthews, 2007).

Chronic maternal stress leads to an increased exposure of the fetus to high levels of endogenous glucocorticoids and this affects the correct programming of the HPA axis, and, therefore, the response to stress. Correlations between maternal and fetal/newborn cortisol concentrations are established during pregnancy and early *postpartum* period (Gitau, Fisk and Glover, 2004; Smith et al., 2011), indicating that maternal glucocorticoids can influence the reactivity of the HPA axis in neonates. Several epidemiological studies have highly correlated stressful events during pregnancy with negative effects on the newborn (for review see Harris and Seckl, 2011;

Reynolds et al 2013), such as spontaneous abortion, preterm birth, low body weight, developmental delays, and long-term behavioral abnormalities (Stott, 1973; Homer, James and Siegel, 1990). However, investigation of the programming role of early-life stress is difficult in humans because of poor retrospective approaches and ethical issues. Accordingly, a variety of prenatal stressors have been used in animal models in order to study late consequences of the manipulation of early life environment and better dissect mechanism underlying stress-related disorders. During the last decades, researchers have developed animal models, in rodents in particular, where stress in applied to pregnant dams or early in life.

a) Early handling paradigm

Since 1957, Seymour Levine developed the "neonatal handling" paradigm that consists in 15 min of daily 'handling' of rat pups during the first week or two of life. This early postnatal manipulation permanently increases GR density in the hippocampus and prefrontal cortex (Meaney et al., 1988). As a consequence, glucocorticoids-induced negative feedback on the HPA axis is potentiated, leading to an overall decrease of stress responsiveness (Meaney et al., 1996). This adaptive process minimizes the risk of damage of the CNS. Also, anxiety-like behavior is decreased in these animals as opposed to neophobia that is reduced (Levine, 1957; Weinberg, Smotherman and Levine, 1978). This animal model is of physiological relevance for maternal behavior, becuse pups are repeatedly but shortly separated from their mother. Indeed, this separation increases maternal care-related behavior, which reduces the activity of the HPA axis and elicits resilience to stress in the adult offspring (Liu et al., 1997; Pryce, Bettschen and Feldon, 2001). Long-term outcomes of early stress programming can be reverted by manipulation in the immediate postnatal environment (Maccari et al., 1995; Vallée et al., 1999; see below). This evidence suggests a crucial role for maternal behavior in the programming processes of the early postnatal period.

b) Maternal separation paradigm

Consistently, long periods of maternal separation during early postnatal life also induce permanent alterations, especially in brain systems important to regulate behavior and stress responsiveness. (Nishi, Horii-Hayashi and Sasagawa, 2014). Depending on the time, duration, frequency and predictability, maternal separation has a different impact. In general, maternal separation causes an increased anxiety-like behavior in the adult offspring associated with a hyper-reactivity of the HPA axis in response to stress (McCormick, Kehoe and Kovacs, 1998; Francis et al., 1999; Huot et al., 2004). Accordingly, 3 hours of daily separation during the first two week induces anxiety- and depressive-like behavior when rodent were exposed to stress in

the adult life (Daniels et al., 2004; Uchida et al., 2010). However, longer interruption of mother-pup interaction may cause adaptive responses in the resilience to stress, as suggested by the evidence that 8 hours of maternal separation per day decreases the response of the HPA axis to stress (Enthoven et al., 2008). Six hours of maternal separation are also sufficient to reduce anxiety-like behavior in response to stressful events in the adult life (Roman et al., 2006).

During development, offspring HPA axis is not equally vulnerable to stress. In the early postnatal period, the rodent HPA axis presents a period of stress hyporesponsiveness (SHRP) that goes from postnatal day (PND) 4 to PND14 in rats and from PND2 to PND12 in mice (Levine, 2001). During this period, basal levels of ACTH and glucocorticoids are lower than normal (Rosenfeld et al., 1991). Maintenance of low levels of stress hormones is crucial for normal growth and development of the brain. Hence, the SHRP is hypothesized to be neuroprotective against stress-induced excessive stimulation of GRs (Sapolsky and Meaney, 1986). In rodents, the presence of the mothers seems to heighten the inhibition of the developing HPA axis and favor SHRP. Disturbances of this process induced by maternal separation generally cause an excessive exposure of the brain to high glucocorticoids levels, with resulting pathological outcomes later in life. There is little doubt that early life experience may have profound consequences on later responses to stress in experimental animals. While brief separations from the dam results in a general stress-resistant phenotype, repeated prolonged separations coupled to an inadequate maternal care and an environment that contains limited stimulation produce a more stress vulnerable organism (Levine, 2005). Thus, the amount as well as the quality of mother-pup interaction is important feature in offspring development.

c) Prenatal stress model

It appears evident that adverse events occurring in this sensitive window might have dramatic long-term consequences in the offspring development. In order to investigate whether exposure to stress earlier during gestation could also induce deleterious effects, different procedures can be adopted to expose pregnant dams to stress condition while pregnant. Rodents are particularly sensitive to glucocorticoids and are very frequently used to investigate long-term effects of early life stress, because their gestation is relatively short (i.e., about 21 days). During the last decades, specific animal models have been developed, especially in rats, where stress is applied to pregnant dams or early in life, and the outcome is examined in the developing offspring and all along their life span. Pregnant dams are typically exposed to

psychological stressor (e.g., repeated restraint, noise or strobe lighting applied on unpredictable basis; Fride and Weinstock, 1984; Henry et al., 1994), physical stressor (e.g. hypoxia or immune challenge; Fan et al., 2009; Paris et al., 2011), or both (e.g., social stress; Bosch et al., 2007; Brunton and Russell, 2010).

Here, I will focus on a particular stress paradigm that has been adopted in our studies, i.e., the perinatal stress (PRS) model that consists in restraint stress during gestation associated with later lower maternal care. This rat animal model has been revised according to the model of Ward and Weisz (1984). Rats exposed to PRS - i.e., the offspring of dams submitted to repeated episodes of restraint stress during the last 11 days of gestation - develop long-lasting biochemical and behavioral changes that likely reflect the induction of a pathological programming induced by early overexposure to glucocorticoids (Maccari et al., 2003; Fig. 13). Interestingly, as discussed above, late gestation corresponds to a fetal HPA activation. In rats, increasing levels of both ACTH and corticosterone are found in fetal plasma in the last gestational week (Boudouresque et al., 1988). Hence, this period is a critical time-window for triggering a perinatal programming induced by glucocorticoids.

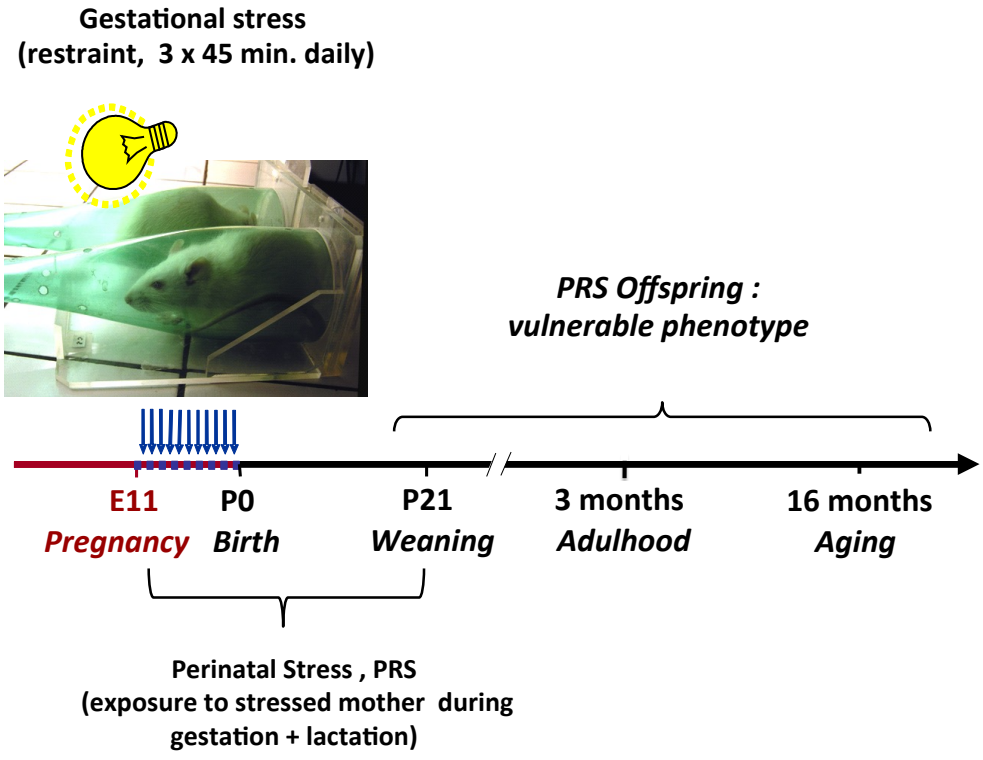

Figure 13: The perinatal stress paradigm in rats.

During the last 11 days of gestation, the dam is exposed to restraint stress under bright light for forty-five minutes, three times per day. No painful contention is applied, but the mother cannot escape to this situation. At birth, the offspring is left undisturbed with the mother until weaning at post-natal day 21 (P21).

2.2. Long-term deleterious effects of early-life stress: the perinatal stress model

2.2.1. Alteration of neuroendocrine homeostasis

a) PRS alters HPA axis activity all life-long

The offspring of dams exposed to gestational stress show an abnormal regulation of the HPA axis. In pre-weaning rats, PRS increases stress-induced corticosterone levels, despite the SHRP (Henry et al., 1994), and leads to a prolonged corticosterone secretion after stress in adulthood (Fride et al., 1986). It has been demonstrated that the impaired activity of the HPA axis is associated with a decreased activation of glucocorticoid receptors in the hippocampus of PRS rats (Weinstock et al., 1992; Henry et al., 1994; Maccari et al., 1995; Barbazanges et al., 1996a; Koehl et al., 1997; Fig. 14).

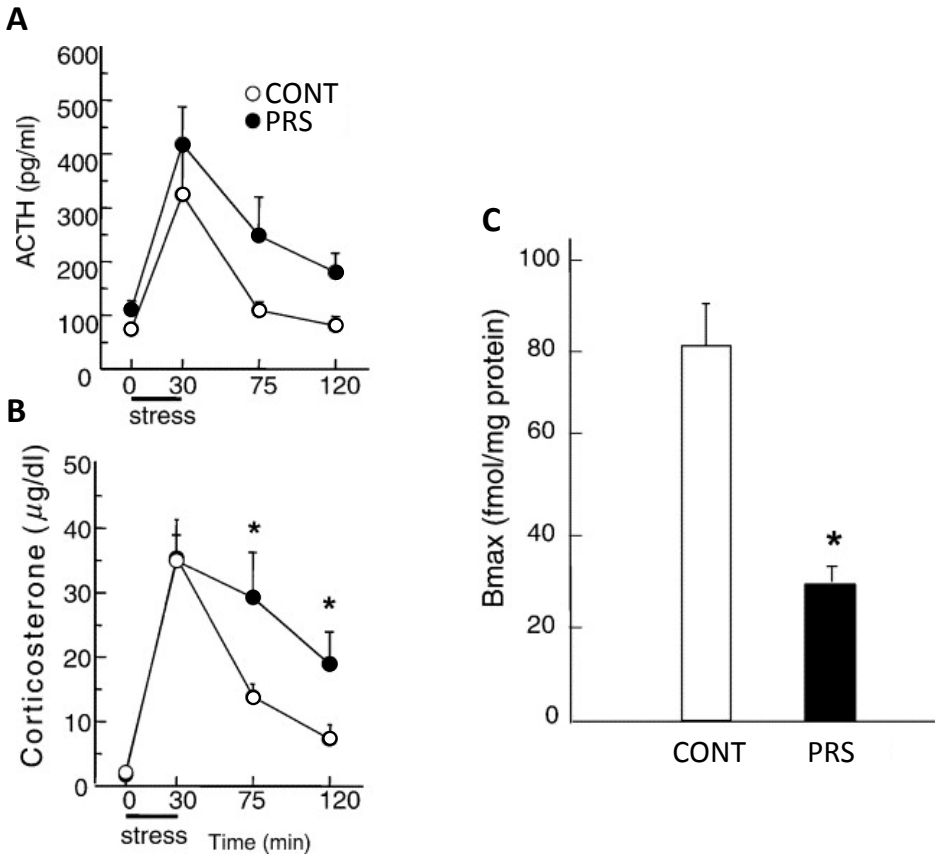

Figure 14: Perinatal stress alters HPA axis response to stress.

(A) ACTH secretion at 4 months in control (CONT) and perinatally stressed (PRS) animals after 30 min of restraint stress. PRS rats show a tendency to have a prolonged ACTH secretion after stress. (B) Corticosterone secretion in CONT and PRS animals. Time-course of the secretion of corticosterone is represented at different times following a 30 min restraint stress (black line of abscissa). The post-stress secretion of corticosterone, at T75 and T120 min, is increased in PRS rats in comparison to CONT. (C) Hippocampal type I corticosteroid receptors. PRS rats present decreased type I receptors (PRS vs CONT, *p<0.05). Error bars show SEM (adapted from Maccari et al., 2003).

Changes in corticosterone levels in response to stress are persistent during entire life span, which suggests that the outcome of PRS on the HPA axis is permanent, albeit not irreversible. Accordingly, PRS rats show an enhancement in glucocorticoids levels during ageing (Henry et al., 1994; Vallée et al., 1999; Fig. 15). All together, these data suggest an impaired glucocorticoid-dependent negative feedback of the HPA axis in PRS rats.

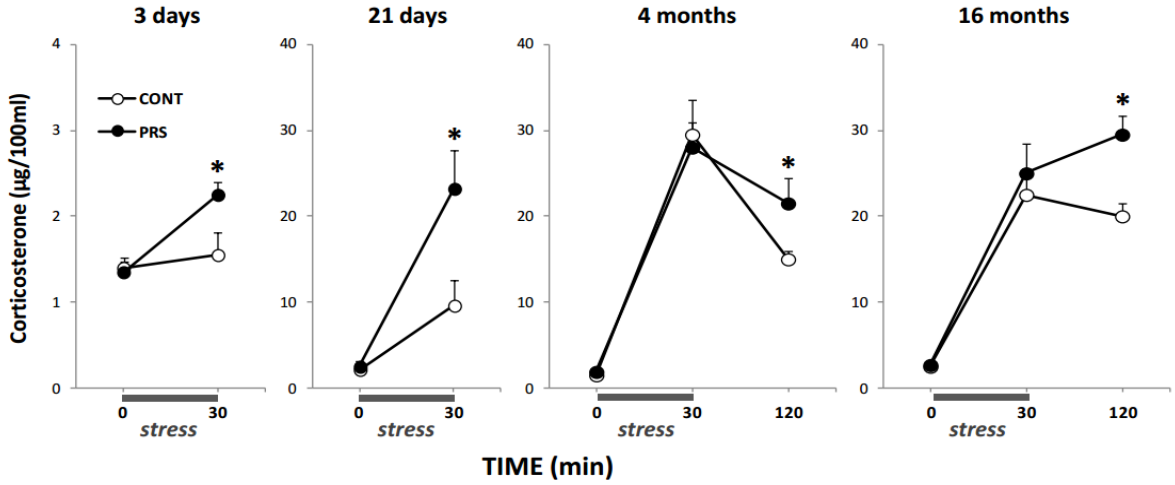

Figure 15: Perinatal stress alters corticosterone response to stress to stress during life span. Plasma corticosterone was measured after 30 min of stress in control (CONT) and perinatally stressed animals (PRS). Values are means ± SEM (adapted from Henry et al., 1994; Vallée et al., 1997; 1999).

b) PRS induces neurochemical modifications

Gestational stress has major effects in the offspring adaption to stress (see Table 3). Stress is tightly associated with activation of the immune system. Interestingly, perinatal stress induces alterations in immune function. Male PRS rats develop a heightened pro-inflammatory state (Vanbesien-Maillot et al., 2007). Norepinephrine is another important factor of the stress response. Within the LC, noradrenergic neurons play a key role in the sympathetic stress response as well as in attention processes (Morilak et al., 2005). PRS rats display increased basal norepinephrine levels in the hypothalamus (Peters, 1982), whereas plasma norepinephinre levels are unchanged (Weinstock et al., 1998). However, circulating norepinephrine levels are increased after footshock stress in PRS rats (Weinstock et al., 1998), suggesting a hyperactive noradrenergic system in these animals, suggesting that early life stress causes a greater response of the noradrenergic system to stress in the adult life. Also, PRS has been reported to induce alteration in the serotonergic system (Peters, 1988a). A reciprocal interaction exists between the HPA axis and 5-HT (Joëls, Hesen and de Kloet, 1991; Meijer and de Kloet, 1998), suggesting that changes in 5-HT system contribute to the hyperactivity of the HPA axis in PRS

rats. PRS causes an increased expression of 5-HT_{1A} receptors in the cortex (Morley-Fletcher et al., 2004) and hippocampus (Hayashi et al., 1998). Interestingly, changes in dopaminergic system have also been demonstrated in PRS rats and this may contribute to increased sensitivity of these animals to psychostimulants (Deminiere et al., 1992; Henry et al., 1995; Koehl et al, 2000; Morley-Fletcher et al., 2004b; Reynaert et al., in preparation).

Recent work from our group investigated glutamatergic and GABAergic systems in PRS rats. PRS rats show a reduced expression of the γ2 subunit of $GABA_A$ receptors in the amygdala (but not in the hippocampus) at PND14 and 22, a reduced expression of mGluR5 in the hippocampus at PND14 and 22, an increased expression of mGluR5 in the amygdala at PND22, and a decreased expression of mGluR2/3 in the hippocampus at PND22 (Laloux et al., 2012). Barros and colleagues (2004) showed increased levels of NMDA receptors are in the cortex, hippocampus and striatum of PRS rats. As discussed above, several lines of evidence indicate that stress-induced rise in glucocorticoids causes changes in glutamatergic transmission in cortico-limbic areas, thereby influencing cognitive and emotional processes. Interestingly, dysfunction of glutamatergic neurotransmission is increasingly considered as an important feature for stress-related mental disorders (Popoli et al., 2012). Work of our group has shown a pronounced gender effect of PRS on mGluR expression as well as on a variety of neurobiological parameters classically associated with hippocampus-dependent behavior. Adult male PRS rats display decreased activity of mGluR1/5 in the ventral hippocampus, increased levels of brain-derived neurotrophic factor (BDNF) ad pro-BDNF, as well as a reduction newly formed granule cells in the DG. Conversely, PRS female rats show an increase in mGluR1/5 activity in the ventral and dorsal hippocampus, with no changes in BDNF levels or hippocampal neurogenesis (Zuena et al., 2008). A recent report demonstrates that changes in sex hormones account for sexual dimorphism found in PRS rats. Accordingly, PRS male rats show reduced levels of testosterone, whereas female rats show reduced of 17β-estradiol (Reynaert et al., 2015).

In the lasts years, work from our group has focused ton he glutamatergic transmission in PRS rats, particularly targeting the ventral hippocampus, a key region in stress-related memory processes (Fanselow and Dong, 2010). Marrocco and colleagues (2012) could demonstrate a causal relationship between selective reduction of glutamate release and anxiety-like behavior in PRS rats using a specific drug cocktail that was able to enhance depolarization-evoked glutamate release in synaptosomes isolated from the ventral hippocampus of PRS rats: a combination of the mGlu2/3 receptor antagonist, LY341495, and the $GABA_B$ receptor

antagonist, CGP52432. The ventral portion of the hippocampus appears to be particularly vulnerable to early-life stress. Indeed, PRS rats also display a reduced neurogenesis in this specific region, which is prominent in males (Zuena et al., 2008; Morley-Fletcher et al., 2011).

2.2.2. Perinatal stress and impairments in affective and cognitive behaviors

a) Anxious-/depressive-like behaviors in PRS rats

Exposure to prenatal stress also induces long-lasting effects on behavior, causing general impairment of the adaptive capabilities of the individual (Weinstock, 2001). Animal studies showed that PRS reduces juvenile play behavior in males to levels normally observed in PRS females (Ward and Stehm, 1991), thus eliminating the gender effect in play behavioral (Meaney, 1989). Evidence that prenatal stress alters social behavior has been also provided in humans. Reduced peer sociability is observed in prenatally stressed children who were in the first half-year of life at the time of war (Meijer, 1985). Along this line, PRS rats show a reduction in play behavior, which is also detectable during adolescence (Morley-Fletcher et al., 2003b) and in the adult life (Weinstock, 2001). Vallée and coworkers (1997) have shown a strong correlation among exploratory capabilities, emotional behavior, and corticosterone secretion after stress. Several studies from Prof. Maccari's group have demonstrated that PRS enhances anxiety-like behavior in the adult life. This was shown in the elevated-plus maze, in which PRS rats spent less time in open arms (e.g. Vallée et al., 1997, Marrocco et al., 2012), as well as in the light-dark box, in which PRS rats spend less time in the light compartment (Ward et al., 2000; Marrocco et al., 2012).

Exposure to chronic stress was shown to decrease sucrose preference (Willner et al., 1987; Moreau et al., 1994), thereby inducing an anhedonic state. Keshet and Weinstock (1995) showed a sexual dimorphism in sucrose preference, which appeared to be decreased in female, but increased in male PRS rats. Learned resignation is a hallmark of depressive-like behavior in rodents, and PRS causes an increase in the immobility time in the forced-swim test in both males (Morley-Fletcher et al., 2003a; 2004a) and females (Alonso et al., 1991).

b) Cognitive impairments in PRS rats

The reduction in neurogenesis observed in PRS rats has been associated with impairments in hippocampal-dependent spatial tasks. Indeed, adult PRS animals show reduced spatial learning abilities in the Morris Water Maze test (Lemaire et al., 2000), as well as in the water-filled T maze (Nishio et al., 2001). Conversely, PRS display retarded performances on the reversal

stage of an appetitive operant discrimination task, with no changes in the acquisition of an operant conditioning (i.e. bar pressing response; Weller et al., 1998).

c) Perinatally stressed rats as a tool for pharmacological investigations of neurological disorders

Perinatal stress appears to be the upstream causative factor for several neurological abnormalities, and the model used in our group turned out to be an interesting tool for the assessment of novel therapeutic strategies. Indeed, the model of perinatal stress in rats is endowed with face, construct and pharmacological validity.

i) *Face validity*: PRS rats display a hyperactivity of the HPA axis (Henry et al., 1994; Maccari et al., 1995; Vallée et al., 1997; Koehl et al., 1997). Also, these animals show dysfunctions in serotoninergic system (Peters, 1998a), as well as changes in sleep architecture (Dugovic et al., 1999) and circadian rhythms (Mairesse et al., 2013). These abnormalities are also reported in depressed patients (Meltzer and Lowy, 1987; Poland et al., 1992). PRS animals also show high levels of anxiety, as frequently observed in depressed patients (Rouillon, 1999). As outlined above, PRS rats show depressive-like behavior in the forced-swim test, and a reduced hippocampal neurogenesis (Lemaire et al., 2000; Zuena et al., 2008; Morley-Fletcher et al., 2011), which is also found depressed patients (Sheline et al., 1996; Sapolsky, 2000; McEwen, 2001). Remarkably, PRS rats show cognitive dysfunctions (Lemaire et al., 2000), which is now considered a core manifestation of depression, and has prognostic value in major depression disorders (reviewed by Goeldner e al., 2013). From a biochemical standpoint, our findings that glutmate release is impaired in PRS rats (Marrocco et al., 2012; 2014) are in line with the "glutamatergic hypothesis" of depression and anxiety (Matrisciano et al., 2007; Hashimoto, 2009; Sanacora, Treccani and Popoli, 2012). Altogether, these findings support the face validity of PRS rats as a model for anxious/depressive disorders.

ii) *Construct validity*: Exposure to a stressful environment during critical periods of brain maturation might be crucial for the development of CNS dicorders later in life. Human studies highlight a strong correlation between decreased hippocampal volume and childhood trauma in depressed women (Vythilingam et al., 2002). Recent findings have also shown a correlation between depressive disorders and childhood maltreatment (Vinkers et al., 2014). These observations reinforce the stress-based theory of mood disorders (Kessler, 1997). Thus, the PRS model has good construct validity. PRS rats also show a reduced body weight at birth (Mairesse et al., 2007a), which may reflect the setting of a fetal programming of metabolic disorders (Gluckman and Hanson, 2004).

iii) *Predictive validity*: Several studies from our group investigated the effects of chronic antidepressants treatments in PRS rats. Remarkably, most of the behavioral and neurochemicals alterations induced by PRS were reversed by chronic antidepressant treatment. Thus, chronic treatment with tianeptine, a drug targeting both the serotonergic and glutamatergic systems (Zhang et al., 2013), or imipramine was able to reduce the immobility time in the forced-swim test (Morley-Fletcher et al., 2003a; 2004a). In addition, imipramine treatment enhanced corticosteroid receptor density in the hippocampus and caused changes in 5-HT_{1A} mRNA levels in PRS rats (Morley-Fletcher et al., 2004a). Treatment with agomelatine, a novel antidepressant, which acting at both melatonin MT1/MT2 and a 5-HT_{2C} receptors, was able to correct abnormalities in the sleep pattern and circadian rhythm in motor activity in PRS rats (Mairesse et al., 2013). This treatment could also correct the abnormalities in mGluR expression and hippocampal neurogenesis in PRS rats (Morley-Fletcher et al., 2011). These data strongly support the predictive or pharmacological validity of the PRS model of anxiety ad depression (see also Chapter 2). Interestingly, exposure to an enriched environment during adolescence was also able to correct the hyper-reactivity of the HPA axis in PRS rats, suggesting that the predictive validity of the model extends beyond pharmacological treatments (Morley-Flecther et al., 2003b).

Table 3: Long-term alterations induced by perinatal stress (adapted from Darnaudéry and Maccari, 2008).

HPA axis dysfunction	Metabolic, immune dysfunctions	Behavioral dysfunctions
Hippocampal MR an GR receptors: ↘ of mRNA in the hippocampus of adolescent males: main effect in the CA3 (Van Waes et al., 2006); ↘ 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 and weight:** ↘ at birth in males (Lesage et al., 2004; Mairesse et al., 2007a); ↗ in adolescent, adult, and 10 month-old males (Lemaire et al., 2000) and in 26 month-old females (Darnaudery et al., 2006). **Corticosterone secretion:** Basal levels: ↘ at birth in males (Lesage et al., 2004); ↗ at the end of the light phase in males and throughout the cycle in females (Koehl et al., 1997, 1999); ↗ in old males (Vallée et al., 1999). After novelty stress (corridor circular, restraint, elevated plus-maze): suppression of the stress hyporesponsive period in infant rats (Henry et al., 1994); ↘ of the sensitivity of the negative feedback of the HPA axis in adolescent, adult and 16 month-old males (Henry et al., 1994; Maccari et al., 1995; Barbazanges et al., 1996; Vallée et al., 1997, 1999; Morley-Fletcher et al., 2003a,b; Viltart et al., 2006). ↗ of the corticosterone response in 26 month-old females (Darnaudery et al., 2006). *After pharmacological stress (alcohol):* ↘ of the HPA response (CRH mRNA, POMC mRNA, corticosterone levels, ACTH levels) after an acute exposure to alcohol (1.5 g/kg, i.p.) in adolescent males (Van Waes et al., 2006).	**Body weight:** ↘ at embryonic day 21 in male and female fetuses (Lesage et al., 2004; Mairesse et al., 2007a,b); ↘ in adolescent males (Van Waes et al., 2006); ↘ in adult males (Vallée et al., 1996). **Glycaemia:** ↘ 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 in22 month-old males (Vallée et al., 1999).

2.2.3 Perinatal stress has persistent effects during aging

A growing body of evidence suggests that the outcome of early life stress is very long-lasting, and, therefore, may critically influence mechanisms of neuroadaptation occurring during aging. If so, PRS might shape the vulnerability to age-related disorders that involve the motor and cognitive domains, such as Parkinson's disease (PD), Alzheimer's disease (AD) and frontotemporal dementia (FTD). Remarkably, depression and anxiety frequently occur since the prodromic phase of these disorders, and their presence complicate the management of PD, AD, and FTD patients.

An increased corticosterone secretion to novelty stress in the youth is predictive of a hyper-reactivity of the HPA axis during aging and may predispose old rats to cognitive decline (Dellu et al., 1996). Vallée and coworkers (1999) showed that old PRS rats exhibit an enhanced corticosterone response to stress associated with memory deficits in the Y maze. Similarly, Darnaudéry and colleagues (2006) have found that 24-month-old PRS female rats show an increased latency to find the submerged platform in the Morris Water Maze as comparison to old unstressed controls or young rats. Old PRS rats also show also metabolic abnormalities, including a hyperglycaemia under fasting conditions and in response to an oral glucose tolerance test (OGTT, Lesage et al., 2004). These results suggest that perinatal stress could increase later vulnerability to metabolic disorders (Fig. 16).

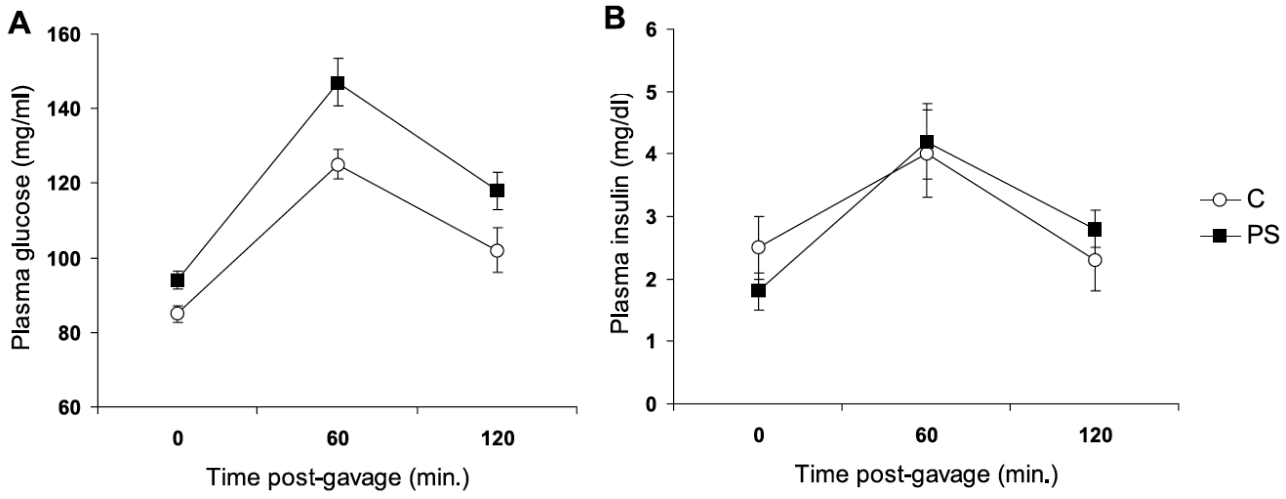

Figure 16: Effect of prenatal stress on plasma glucose (A) and insulin levels (B) during an oral glucose test tolerance (OGTT) in 24-month-old controls (C) and perinatally stressed (PS) rats (from Lesage et al., 2004).

These results suggest that perinatal stress leads to long-term disturbances in adaptation to stress life span. These animals develop an important "allostatic load" during life and coping with challenges represent a high cost process. This induces the development of increased anxiety, depressive-like behavior and cognitive impairments. Nevertheless, persistent effects of perinatal stress during aging have been investigated only in a few studies.

2.3. Early post-natal intervention corrects PRS-induced abnormalities

As discussed above, perinatal stress may have long-term deleterious effects on the individual. One expects that early interventions, preferentially before weaning, may restrain the development of the "pathologic" programming predisposing stressed individuals to CNS disorders in the adult life. Exposure to glucocorticoids either prenatally and/or soon after birth is considered one important factor in the development of the pathological programming in response to perinatal stress. Barbazanges and colleagues (1996a) showed adrenalectomy in pregnant dams exposed to restraint stress prevented the long-term consequences of gestational stress in the adult offspring. Interestingly, restraint stress delivered to pregnant dams reduces 11β-HSD2 activity in the placenta, thus increasing the amount of non-metabolized corticosterone reaching the fetus (Mairesse et al., 2007a).

The early postnatal period also plays a crucial role in programming the reactivity of the offspring to stress in the adult life. Previous work by Maccari and coworkers (1995) showed that early adoption of PRS rats by unstressed mothers was able to reverse HPA abnormalities normally found in PRS rats (Fig. 17). This suggests that many facets of the long-term outcome of PRS might be reversed by pharmacological or behavioral manipulations performed in the early postnatal period. Of note, the beneficial effect of adoption on the PRS phenotype was seen only if adoption was performed in the first hours after birth (Barbazanges et al., 1996b). Adoption *per se* has an important effect on maternal behavior. Foster mothers spend more time with pups than biological mothers (Maccari et al., 1995; Fig. 17). In addition, abnormalities in dopaminergic and glutamatergic transmission induced by PRS are also corrected by early adoption (Barros et al., 2004). Recent work also showed that early adoption of PRS rats by control dams could correct the reduction in the number of oxytocin- and vasopressin-positive neurons in the PVN of the hypothalamus as well as the impairment in anxiety-like and social behaviors (de Souza et al., 2013; Fig. 18). AVP and oxytocin are known to modulate social behavior in a variety of animal species and humans (Ebstein et al., 2012; Lukas and Neumann, 2012; Young et al., 2011). In rodents, oxytocin and vasopressin influence aggressive (Bosch et

al., 2005), affiliative (Goodson et al., 2009) and sexual behavior (Winslow et al., 1993), in addition to social interaction ad memory (Todeschin et al., 2009; Tobin et al., 2010). Thus, changes in the levels of AVP and oxytocin might have a role in the programming induced by PRS stress. This evidence laid the groundwork for a series of studies that will be presented in the experimental session.

Manipulation of the early environment using an early handling paradigm (discussed above) was also efficient in reversing the deleterious effect of PRS in the offspring. Important work from Monique Vallée highlighted that prenatal and postnatal manipulations had opposite effects. Early handling of adult PRS rats was able to enhance basal feeding behavior and body weight but had only little effect on blood glucose levels that are elevated in these animals (Vallée et al., 1996). Also, this paradigm could reduce corticosterone response to stress in adult, middle-aged and old PRS rats (Vallée et al., 1999). From a behavioral standpoint early handling also reduced anxiety-like behavior (Vallée et et al., 1997). Whether the effects of handling are du to sensory stimulation of the pups or to the brief and repeated interruptions of maternal care remains to be established.

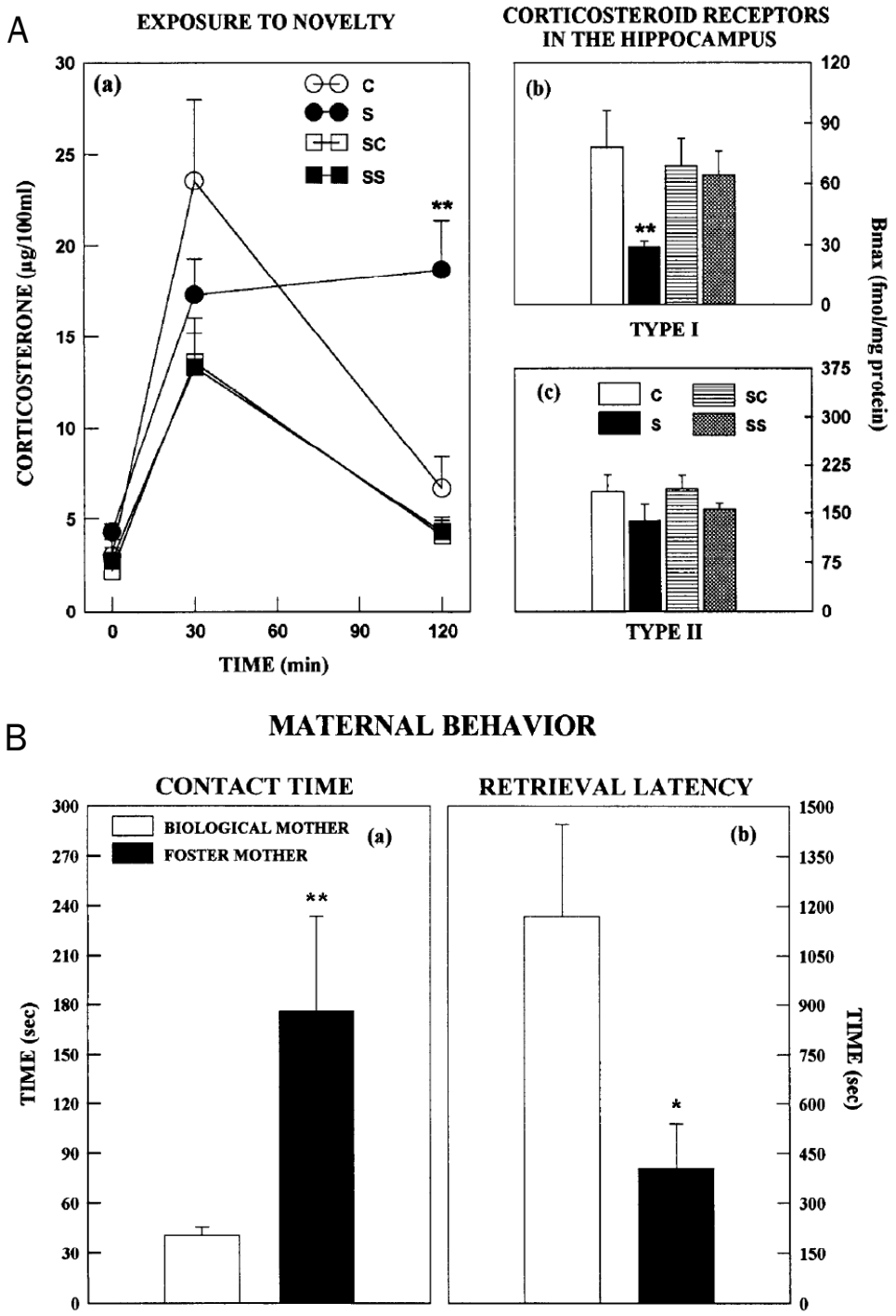

Figure 17: Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress.

(A) Plasma corticosterone secretion after novelty exposure (a) and type I (b) and II (c) corticosteroid receptors in the hippocampus of adult prenatally unstressed rats raised by their biological mother (C), adult prenatally stressed rats raised by their biological mother (S), adult prenatally stressed rats adopted by a control unstressed mother (SC), and adult prenatally stressed rats adopted by a mother stresses during gestation (SS). (B) Effects of adoption on maternal behavior. a, Foster mothers spent longer licking and picking up the pups than did biological mothers. b, Latency to replace all the pups in the nest was lower in foster than in adopted mothers (adapted from Maccari et al., 1995).

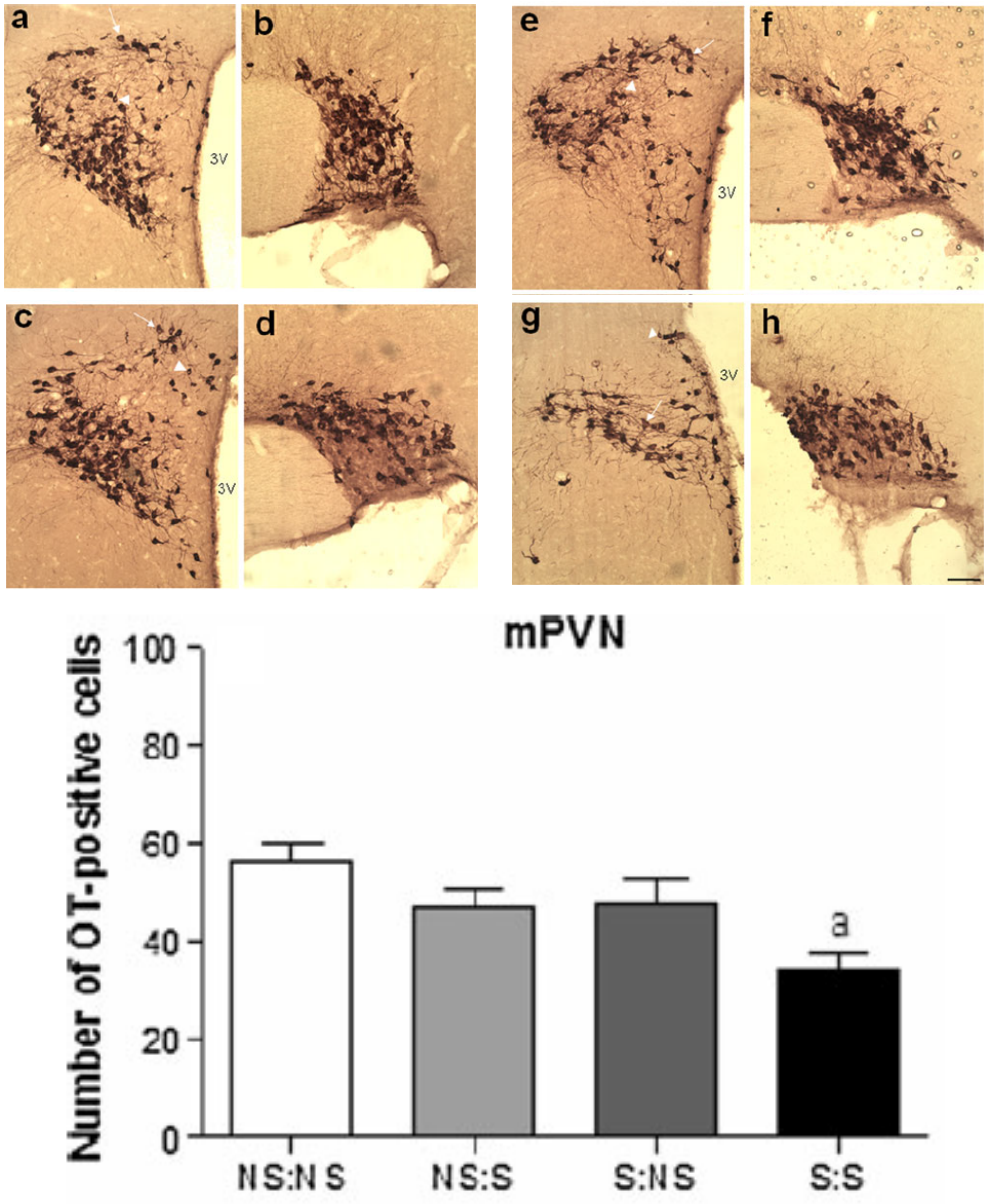

Figure 18: Representative photomicrographs of oxytocin-positive cells in the paraventricular (b, d, f, h) and supraoptic nuclei (a, c, e, g) of each animal group and number of magnocellular oxytocin-positive neurons of the PVN nucleus.

Oxytocin-positive neurons were analyzed in prenatally non-stressed offspring raised by prenatally non-stressed mothers (NS:NS; a and b), prenatally non-stressed offspring raised by prenatally stressed mothers (S:NS; e and f), prenatally stressed offspring raised by prenatally non-stressed mothers (NS:S; c and d), prenatally stressed offspring raised by prenatally stressed mothers (S:S; g and h). Arrowheads parvocellular immunostained neuron, arrows magnocellular immunostained neuron, 3V third ventricle. Magnification—2009. Scale bar 50 µm, a indicates $p<0.05$ when compared to the NS:NS group (adapted from de Souza et al., 2013).

2.4. Epigenetic machinery and perinatal stress-induced reprogramming

2.4.1. Genetics vs epigenetics: a dynamic interplay

Individual variations in the vulnerability to develop stress-related disorders depend on an intricate interplay between the genetic background and the impact of the environment. Environmental factors may cause permanent changes in gene expression, which are mediated by mechanisms of DNA methylation and histone acetylation *inter alia* (see below). These mechanisms might predispose the individual to develop age-related disorders (Huidobro, Fernandez and Fraga, 2013), and, interestingly, might also be transmitted trans-generationally. This raises the interesting possibility that early life stress causes changes in the reactivity of the HPA axis that can be transmitted to the progeny even for more than one generation. Historically, the word "epigenetics" was used to describe events that could not be explained by genetic principles. Conrad defined epigenetics as "*the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being*" (Waddington, 1942). Nowadays, epigenetics may be defined as the study of any potentially stable and heritable modification in gene expression or cellular phenotype that occurs without changing the underlying DNA sequence (Goldberg, Allis and Bernstein, 2007).

2.4.2. Epigenetic profile inheritance

Investigations in the epigenetics field have significantly clarified the mechanisms underpinning the programming effects of stress (McEwen et al., 2012). Epigenetic alterations, initiated during the life of our ancestors and during different life stages of our own existence, in tandem with maternal phenotypic effects, can contribute to development of a premature aging phenotype, or facilitate physiological senescence through their modulating potential (Lupu, Tint and Niculescu, 2012). Epigenetic mechanisms primarily occur at the chromatin level, and involve multiple mechanisms including DNA methylation, covalent posttranslational modifications of histones (HPTMs), chromatin folding and attachment to the nuclear matrix, and/or nucleosomes repositioning. MicroRNAs and other species of non-coding RNAs are also main actors of epigenetic mechanisms. All these mechanisms act separately or in synergy to modulate chromatin structure and its accessibility to the transcriptional machinery. Generally, DNA methylation on cytosine residues ($-CH_3$) of CpG islands reduces gene transcription, while histone acetylation at specific lysine residues ($-CO-CH_3$) activates gene expression (Zhang and Meaney, 2010).

Gene regulation by epigenetic processes is an important feature to be considered when studying the environmental factors that affect parent generation, because they might contribute to shaping the phenotype of the next generation(s). Interestingly, both glucocorticoids and maternal behavior persistently alters epigenetic marks (Fig. 19). The first evidence for a role of epigenetic mechanism in the long outcome of early life stress was provided by studies of maternal care in rats. Weaver and colleagues (2004) showed that the extent of maternal care, evaluated as the time spent in licking and grooming (LG) and arched-back nursing (ABN), was a critical determinant in the epigenetic regulation of the GR-encoding gene in the hippocampus of the offspring. The offspring of mothers displaying low maternal care during the first week after birth ("low-LG and ABN mothers") showed a reduced GR expression in the hippocampus and a heightened response to stress. In these animals, hypermethylation in the promoter region of the GR-encoding genes restrained the efficiency of egr-1 in activating gene transcription. Changes in GR expression were reversed by cross-fostering and persisted across life. More in general, perturbed maternal behavior caused by chronic unpredictable pup-mother separation or maternal stress widely affects methylation in the offspring's brain causing either hypomethylation or hypermethylation of different gene promoters. Strikingly, the aberrant methylation is perpetuated across successive generations and is present in the germline of first-generation males and the brain and germline of second-generation progeny. These changes are associated with multiple stress-related symptoms such as depressive-like behaviors, and social anxiety (Franklin et al., 2010; Weiss et al., 2011). Aberrant DNA methylation due to disrupted maternal care thus affects several tissues, can subsist after meiosis in male germ cells, and is transmitted transgenerationally, suggesting a powerful potential strategy for the maintenance and inheritance of the effects of early chronic stress. Similarly to sperm cells, oocytes may carry epigenetic anomalies in response to stress because transgenerational inheritance of stress-induced symptoms may occur through the female lineage independently of maternal care (Weiss et al., 2011).

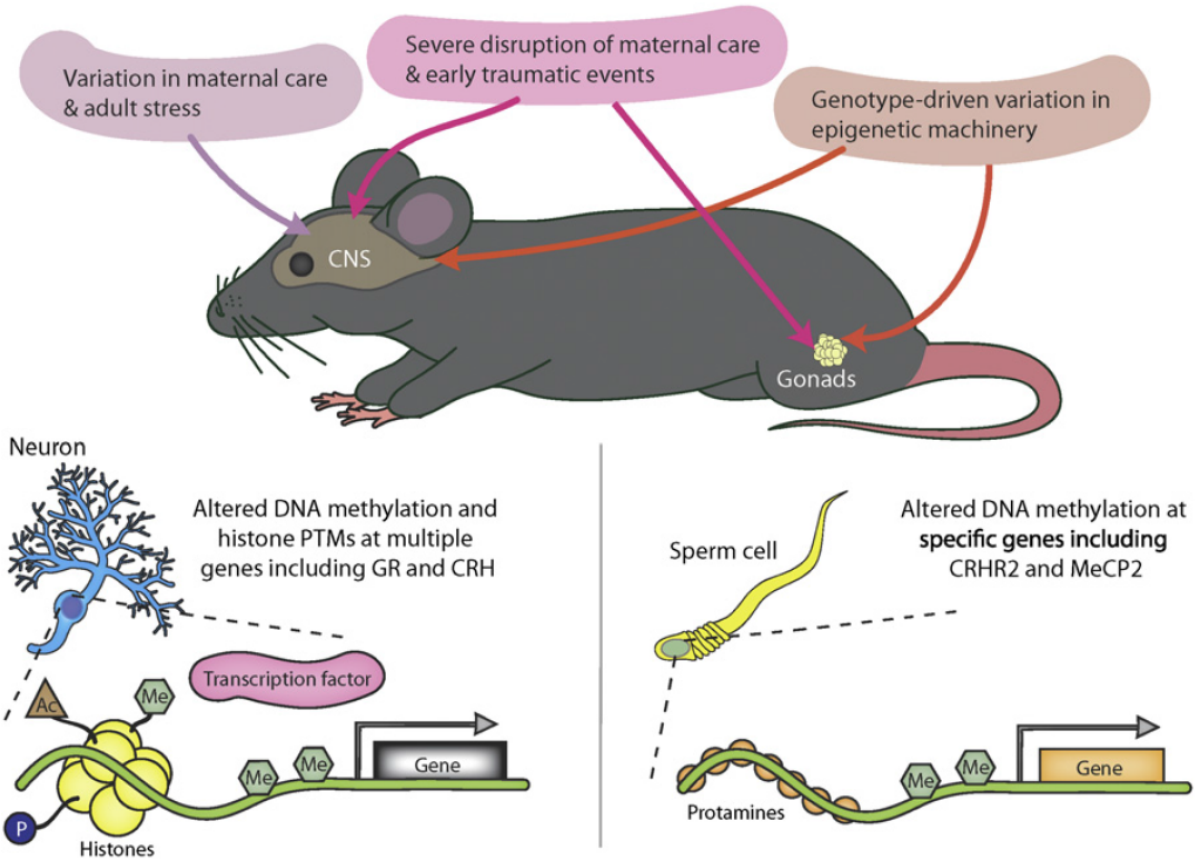

Figure 19: Epigenetic Processes Associated with Stress Responses

Schematic representation of the influence of maternal care on the epigenome in the brain and germline. In the brain, DNA methylation and covalent posttranslational modifications (PTMs) of histones, i.e., histone acetylation (Ac), methylation (Me), or phosphorylation (P) modulate chromatin structure and allow transcription factors to be recruited for transcriptional activation of specific genes such as GR and CRH. In sperm cells, DNA methylation marks specific genes for future transcriptional regulation in the developing and adult animal (from Franklin, Saab and Mansuy, 2012).

Recent work from our group has given evidence for the involvement of epigenetic factors in the programming of stress-related disorders by PRS (Maccari et al., 2012). In particular, a transgenerational effect of PRS has been demonstrated, with transmission across generations of an anxious phenotype and stress-related neurobiological disturbances as well as a transgenerational transmission of gestational stress on mother's behavior and physiological parameters in the offspring with an intrauterine growth restriction. Remarkably, these effects were observed despite the fact that successive offspring was not exposed to stress ("stressed grandmother effect"). Alterations induced by PRS are thus persistent over life span and generations. That epigenetic mechanisms lie at the core of these alterations is suggested by the evidence that PRS induces persistent changes in gene expression that are maintained across generations.

Nevertheless, as opposed to genetic changes, DNA methylation and/or histone acetylation are reversible biochemical reactions. Weaver and colleagues (2005) examined whether epigenetic changes induced by early stress could be reversible by pharmacological treatment in the adult life. For example, central infusion of the histone deacetylase inhibitor, trichostatin A, could correct the epigenetic abnormalities induced by low levels of LG and ABN in the offspring (Szyf et al., 2005). These results demonstrate that, despite their stability, the epigenetic marks established early in life can be reversed by appropriate interventions in the adult life.

2.4.3. O-*GlcNAc: potential role in PRS epigenetic reprogramming*

The high levels of glucocorticoids associated with exposure to stress may cause an impairment of energetic homeostasis in hippocampal neurons, perhaps by reducing glucose utilization. This may eventually result into synaptic dysfunction, cytoskeleton abnormalities,and cell death (Osborne et al., 2015). Nutrient availability may also influence the epigenetic regulation of gene expression. In particular, *O*-GlcNAcylation occurring at the level of chromatin and transcription factors (Kelly and Hart, 1989; Kelly, Dahmus and Hart, 1993) caters the potential to act as a linking bridge between nutrient availability and regulation of gene expression.

O-GlcNAcylation was initially described as a dynamic and reversible post-translational modification that occurs in all cellular compartments. *O*-GlcNAc is an end point of the hexosamine biosynthetic pathway (HBP). Two to 5% of glucose entering the cells is metabolized *via* the HBP into uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). *O*-GlcNAc is transferred to serine and threonine residues of target proteins by the enzyme by *O*-GlcNAc transferase (OGT). Removal of *O*-GlcNAc from proteins is catalyzed by the enzyme, *O*-GlcNAcase (OGA). Synthesis of UDP-GlcNAc is at the crossroad of nearly every metabolic pathway in the cell, and *O*-GlcNAc glycosylation is a key process in cellular signaling and transcription regulatory pathways in response to nutrients and stress (reviewed by Hart et al., 2011; Fig. 20). The cycling of *O*-GlcNAc is essential for vertebrate development, as deletion of the OGT-encoding gene, *Ogt,* is lethal for the developing embryo (reviewed by Love, Krause and Hanover, 2010). Interestingly, *O*-GlcNAcylation plays also an active role in neurologic and metabolic disorders (Issad, Masson and Pagesy, 2010; Bond and Hanover, 2013), and impairments in metabolism and *O*-GlcNAc levels would be involved in the pathogenesis of neurodegenerative disorders (Lefebvre et al., 2003a; 2005). *O*-GlcNAc glycosylation may thereby represent a linking bridge between metabolic disorders, such as diabetes or metabolic syndrome, and neurodegenerative disorders (Lefebvre et al., 2010), particularly in AD (see below; Lefebvre, 2012).

Because glycosylation is one of the most abundant post-translational modifications in eukaryotic cells, it has been suggested that the carbohydrate-processing machinery may interface with the epigenetic control of gene expression at multiple levels (Hanover, Krause and Love, 2012). A recent study demonstrates that histones are *O*-GlcNAcylated (Sakabe, Wang and Hart, 2010) and that levels of *O*-GlcNAcylation vary during cell cycle (Zhang et al., 2011). Thus, *O*-GlcNAc seems to be highly involved in chromatin conformation during transcription and cell cycle progression (Hanover, 2010). It was also reported that OGT and *O*-GlcNAc control the activity of histones methyltransferases and and may regulate acetylation of the histone tails (reviewed by Dehennaut, Leprince and Lefebvre, 2014). Thus, *O*-GlcNAcylation appears to play a key role in chromatin dynamics, thus contributing to mechanisms of epigenetic regulation. In addition, OGT is able to control gene expression more finely by modulating the interaction between gene promoters and protein complexes including transcription factors, co-activators and co-repressors. In particular, Li and coworkers (2012) showed that OGT interacts with the GR protein in the nucleus to repress gene expression. Thus, *O*-GlcNAc can be considered as a key player in the stress-related epigenetic programming.

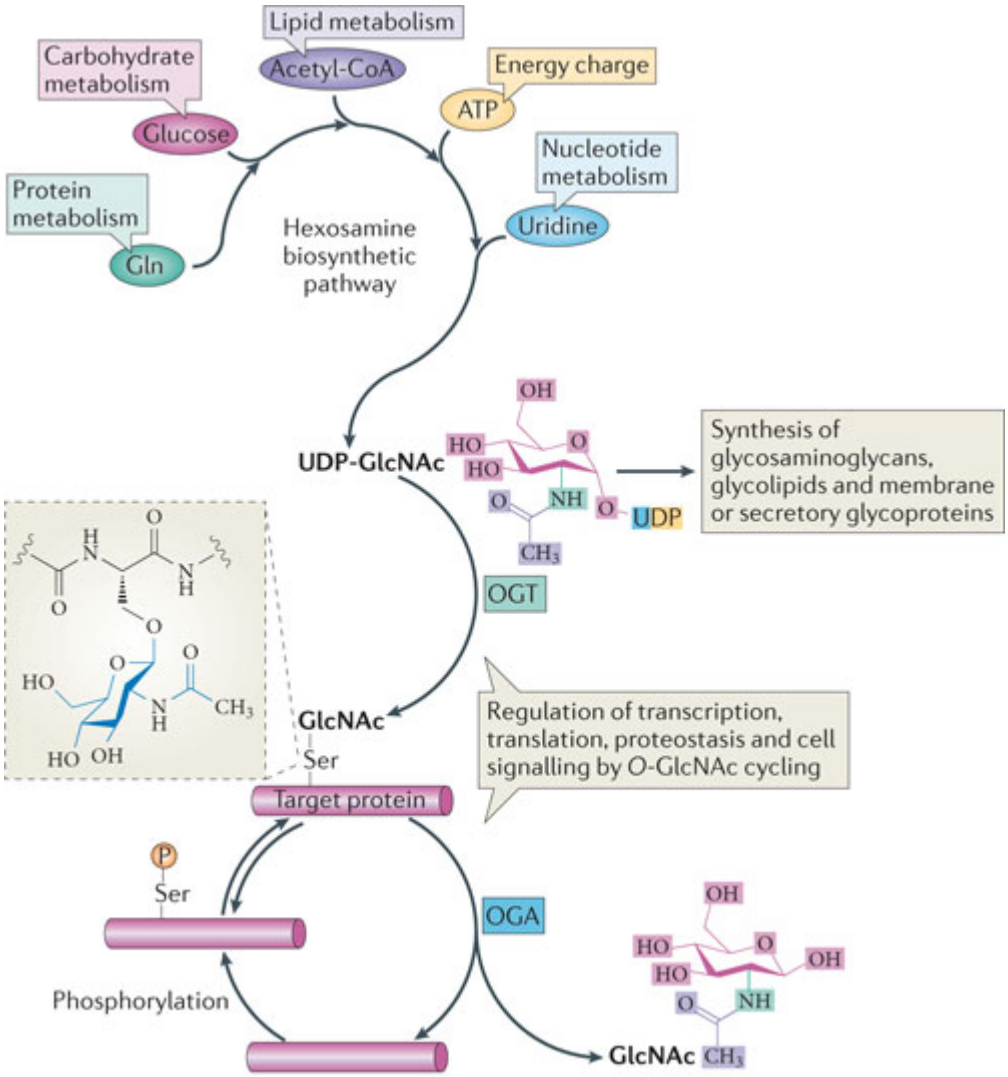

Figure 20: The synthesis of UDP-GlcNAc via the hexosamine biosynthetic pathway is nutrient responsive.

The hexosamine biosynthetic pathway (HBP) integrates the metabolism of carbohydrates, amino acids, fat and nucleotides in the synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is utilized for the synthesis of glycosaminoglycans, glycolipids and membrane and secretory glycoproteins. In addition, it is utilized by *O*-GlcNAc transferase (OGT) in the nucleus and the cytoplasm for the addition of GlcNAc at Ser or Thr residues (not shown) of target proteins (O-GlcNAcylation). Addition and removal of O-GlcNAc by OGT and *O*-GlcNAcase (OGA), respectively, contributes to continuous *O*-GlcNAc cycling, which dynamically modifies numerous intracellular proteins. *O*-GlcNAcylation often occurs at sites that are also targeted by phosphorylation. Thus, the resulting diversity of *O*-GlcNAcylated and phosphorylated proteins might contribute to the regulation of several complex cellular processes, including transcription, translation, proteostasis and cell signalling (from Hanover, Krause and Love, 2012).

3. Stress and maternal behavior: crucial role of oxytocin

Children inherit not only genes from their parents but also an environment
West and King 1987

3.1. Parents vs. offspring: a matter of health

3.1.1. Parental factors are an interface for offspring development

The early postnatal period is critical for the survival of the individual because in early infancy the offspring almost entirely depends on parents or caregivers. Especially in rodents, maternal behavior has a crucial role on neural development. Indeed, during the first week of life, interaction with the dam is the main environmental stimulus perceived by pups. The mother thus represents a direct link between the environment and the developing animal. Parental care during early life heavily contributes to shape the cognitive and emotional development in the offspring. Several human studies highlight an important relationship between child mistreatment and increased vulnerability for illness later in life. The emotional status of the parents is also important for the development of the offspring. Children developing in adverse conditions, e.g. low parental bonding or stressed parents, have increased vulnerability to mental disorders and chronic metabolic and vascular disorders (reviewed by Meaney, 2001).

Studies performed by Michael Meaney and his Associates have shown that lactating rats housed under identical environmental condition, show variations in maternal care. These rats display either low or high maternal care as shown by the analysis of specific behavior, such as LG and ABN (Caldji et al., 1998). These behaviors are good indicators of the maternal recognition and care of their pups (Stern, 1996), and critically influence urination and defecation of the pups (Rosenblatt and Lehrman, 1963). The lack of tactile stimulation at birth is directly associated with reduced survival of the pups in rats (Pederson and Blass, 1982). Meaney and Associates have demonstrated that a reduced maternal care soon after birth causes long-lasting alterations of the HPA response to stress as well as other neurochemical and behavioral consequence in the offspring. The offspring from low-LG mothers show more fearful behavior and express lower GR levels in the hippocampus, resulting into an impaired negative feedback regulation of the HPA axis (Liu et al., 1997). This reflects a pathological epigenetic programming in which DNA hypermethylation causes a reduced transcription of NC3R1 gene encoding for the GR in the hippocampus of the offspring (Weaver et al., 2004). Thus, in addition to maternal glucocorticoids, maternal behavior also plays a critical role in the development of the stress response and related behaviors during the postnatal period (Francis et

al., 1999a; Levine, 2002). In other words, the brain is very sensitive to these perinatal programming factors, and both maternal behavior and glucocorticoids have powerful brain-programming properties (Seckl, 1998). Hence, parental factors mediate the effects of environmental adversity on development. In particular, as discussed above, early life is a critical period for the development of neuronal circuits that mediate the behavioral and neuroendocrine response to stress.

Interestingly, Francis and coworkers (1999b) showed that differences in maternal behavior and reactivity to stress were transmitted across generations. Thus, the female offspring of high-LG mothers displayed a higher LG-ABN behavior with respect to the female offspring of low-LG mothers, and early handling could reverse the developmental trajectory of the phenotype. Thus, the quality of maternal care provided to the offspring early in life modulates the developmental programming of the HPA axis.

3.1.2. Stress responsiveness and peripartum period

Gestational period is a time of dramatic changes in the CNS of the mother, which facilitate the coping with pregnancy and ensure survival of the offspring. Both gestation and lactation are associated with remarkables changes in the regulation of the HPA axis. The response of the HPA axis to stress is reduced from mid-pregnancy through the end of lactation (Stern, Goldman and Levine, 1973; Douglas and Russell, 1994; Neumann et al., 1998; Lightman et al., 2001; da Costa et al., 2001), although lactation in rodents is associated with a chronic elevation in plasma corticosterone levels (Windle et al., 1997b) and with a disruption of the circadian rhythm of glucocorticoid secretion (Lightman et al., 2001). Interestingly, CRH expression is reduced in the amygdala of lactating dams (Davis and Whalen, 2001). Changes in stress-related hormones observed during the *peripartum* period may contribute to the typical phenotype of lactating mothers, which includes reduced anxiety (Windle et al., 1997b; Neumann, 2003), enhanced maternal behavior (Pedersen et al., 1991) and aggressiveness (Gammie et al., 2004; reviewed by Bosch, 2015). These behavioral changes are crucial for the establishment of a secure environment for the offspring and are adaptive modifications essential for the healthy development of the offspring.

Animal research shows that exposure to stressful events during gestation bidirectionally impairs mother-pup interaction. Prenatally stressed pups evoked less maternal care from unstressed foster dams, and stressed dams showed less maternal care towards foster pups than unstressed dams (Moore and Power, 1986). Measurement of ultrasonic vocalizations (USVs) is

a good indicator of stress-induced anxiety in pups. Depending on the type and extent of gestational stress, USV emission in PRS pups at PND14 is either reduced (Morgan et al., 1998) or increased after maternal separation (Williams, Hennessy and Davis, 1998). Investigating USV emission using a "maternal potentiation" paradigm (in which pups are isolated for 5 min, reuinited with their mothers for 1 min, and isolated again for 5 min), Laloux and colleagues (2011) have shown that isolated PRS pups emitted more USVs, whereas a reduced USV emission was found after maternal reunion. The difference between controls and PRS pups was no longer observed after the first two weeks after birth. Changes in USV emission strongly suggest that there are differences in maternal behavior between stressed and unstressed dams. These findings combined with the observation that USV suppression by exposure to unfamiliar male odor is still observed in PRS pups at PND14 (but not in age-matched control pups) indicate a prolonged defensive and anxiety-like behavior in PRS pups beyond the physiological timeframe. Consistently, this indicates changes in the development of the HPA axis in these animals.

Several lines of evidence indicate that maternal care can shape endocrine functions in the pups. Work from Seymour Levine (1994) highlighted that the presence of the mother is a *sine qua non* condition for maintenance of the SHRP in pups, indicating that tactile stimulation by the mother dampens HPA activity in pups. On the contrary, pups exposed to maternal separation have increased glucocorticoids levels. Accordingly, unstressed control animals are hypo-responsive to stress during the first two weeks of postnatal life (de Kloet et al., 1988), while PRS sensitizes the HPA axis increasing stress-induced corticosterone secretion in pre-weaning rats (Henry et al., 1994).

Stress reactivity of the offspring mirrors that of their mothers. Low-LG mothers are more fearful than high-LG mothers, and their offspring exhibits the same phenotype (Francis et al., 2000). Similarly, dams exposed to chronic restraint stress during the last 11 days of gestation show anxiety-like behavior and changes in their reactivity to inescapable stress such as novelty or forced-swim tests (Darnaudéry et al., 2004). These effects persist more than one month after gestational stress suggesting that pregnancy is a period of high vulnerability to stress for both the dams and the offspring.

Stressful situations result into increased plasma corticosterone levels in pregnant rats. Interestingly, gestational stress increases behaviors not directed towards pups (e.g., exploration or self-care) at the expenses of maternal behavior (Patin et al., 2002). Thus, gestational stress makes mothers less pup-oritented.

3.2. Mother/pups bonding and the oxytocinergic system

While HPA reactivity is decreased during gestation, other systems are important for the onset of maternal behavior. Several integrated neuroendocrine mechanisms involving CRH, vasopressin, oxytocin and prolactin play an important role in physiological and behavioral changes associated with maternal care, and are also involved in the endocrine response to stress (see above; Nephew and Murgaroyd, 2013). Pregnancy is characterized marked and sustanined elevations in protegesterone levels associated with milder increases in estrogen levels. Prior to parturition progesterone levels fall and estrogen levels further increase. Both events are compulsory for the onset of maternal behavior. The influence of ovarian hormones on the onset of maternal behavior in rats appears to be mediated, in part, by effects on central oxytocinergic system (Pedersen, 1995). Remarkably, increases in estrogens levels induce OTR gene expression and enhance oxytocin receptor binding (de Kloet, Voorhuis and Elands, 1986; Bale, Pedersen and Dorsa, 1995; Young et al., 1997) in brain region known to control maternal behavior in rats (i.e., medial preoptic area of the hypothalamus and ventral BNST) (Numan, 1994; Numan and Sheehan, 1997). Interestingly, activation of the OTR in the *Nucleus Accumbens* induces a reward signaling (Dölen et al., 2013) that attributes salience to maternal behavior (Olazabal and Young, 2006).

Oxytocin has an established role in the onset of maternal behavior (Bosch and Neumann, 2012). Oxytocin produced by SON and PVN neurons and secreted promotes labor and milk ejection. Milk production involves other hormones, including prolactin, which may also have a role in maternal behavior (reviewed by Mann and Bridges, 2001). Recently, a great emphasis is placed on the action of oxytocin in the CNS. Interestingly, intracerebroventricular administration of oxytocin stimulates maternal behavior in ovariectomized virgin rats (Pedersen et al., 1982), and deficts in maternal behavior are observed in OTR knock-out mice (Takayanagi et al., 2005, Rich et al., 2014). In addition, expression of OTRs has been linked to natural variations in maternal behavior during the *peripartum* period (Insel, 1990), and, in rodents, high levels of OTRs in the central nucleus of the amygdala have been associated with high levels of maternal care (Francis, Champagne and Meaney, 2000; Champagne et al., 2001; Francis et al., 2002). In addition, Champagne and Meaney (2006) have shown that early exposure to environmental adversities during gestation alters both OTR levels and maternal behavior.

Aims of the thesis

Nowadays, stress is recognized as a risk factor for a variety of disorders during life span of the individual. In particular, prolonged response to stress might be the upstream causative factor for disease (McEwen, 1998). Vulnerability to develop chronic diseases may be programmed early in life, and, in particular, during the fetal period, which is critical for shaping the lifelong health of an individual (Barker, 1995). This led to the elaboration of the theory of the developmental origins of health and disease (DOHaD). Mounting evidence indicates that changes in the perinatal environment induce long-lasting effects in the individual from birth to aging (Lupien et al., 2009; Vallée et al., 1999). Exposure to stressful events in this critical period can lead to the development of several neurological, metabolic and neuroendocrine abnormalities in the offspring (Seckl, 1998; Lupien et al., 2009). These kinds of alterations arise because of the permanent organization or imprinting of fetal physiological systems including the CNS, a process known as early programming. This programming particularly involves maternal glucocorticoids. After stress exposure, maternal glucocorticoids released in the bloodstream travel through the placenta and "program" the fetus to the development of stress-related disorders by inducing permanent changes in the regulation of the HPA axis and in the functioning of brain regions that are critically involved in the regulation of the stress response, such as the hippocampus (Seckl and Meaney, 2004). Accordingly, chronic prenatal stress has been shown to induce structural and functional changes in the hippocampus (Lupien et al., 2009; Morley-Fletcher et al., 2011; Marrocco et al., 2012), which in turn cause abnormalities in cognitive and emotional behaviors. Thus, early-life stress is a major risk factor for the vulnerability to psychiatric disorders, such as anxiety and depression, two pathologies associated with cognitive impairments.

During *peripartum* period, both prenatal (Barbazanges et al., 1996) and postnatal factors (Peters, 1988b) have a crucial role in the programming of the developmental trajectory of the offspring. Glucocorticoids play a crucial role in fetal development, ensuring developmental switching in many organs that are essential for life after birth. Nevertheless, stress-induced increases in maternal glucocorticoid levels during gestation are detrimental. As widely discussed above, exposure to high levels of glucocorticoids during critical period of development results in the (re)programming of a pathological phenotype with major consequences in the life span of the individual (Darnaudéry and Maccari, 2008). In early infancy, the mother represents an important interface with the surrounding environment.

Several studies indicate that maternal behavior is an important feature for the development of the offspring, and particularly for the development of neuroendocrine systems that underlie HPA axis and behavioral responses to stress (Francis and Meaney, 1999). Maternal adversities might thus predict impairment in fetal growth and HPA axis reactivity (reviewed by Meaney, Szyf and Seckl, 2007). Maternal exposure to stressful events during gestation induces alteration in the mother-pup interaction, particularly decreasing maternal care in stressed dams (Patin et al., 2002). Oxytocin system is one of the endocrine systems particularly involved in the onset of maternal behavior (Pedersen et al., 1982), and shaping the CNS in the early life.

The model of perinatal stress in rats developed by Prof. Maccari (Maccari et al., 1995), in which pregnant dams are exposed to multiple episodes of restraint stress nicely recapitulates the key features of stress-related disorders and is endowed with face, construct, and predictive validity. The offspring of stressed dams (here, called perinatally stressed rat or "PRS" rats) develop an anxious/depressive phenotype associated with a hyper-reactivity of the HPA axis to stress in the adult life. Nevertheless, few studies have investigated in this model the long-term effects of the joint implication of prenatal restraint stress and early postnatal intervention in the offspring. The aim of the present thesis is **to establish whether these changes could be extended to the old age of the offspring, and, more important, whether the pathological phenotype of PRS rats could be reversed or prevented by pharmacological activation of oxytocin receptors in lactating mothers or in the adult offspring.**

Moving from the evidence of the crucial role of *peripartum* environment and maternal behavior in the developmental trajectory of the offspring, in **CHAPTER ONE**, I investigated the linking bridge between impaired maternal environment during gestation and detrimental consequences in the offspring during life span. In particular, I focused on maternal oxytocinergic system and investigated whether stimulation of maternal OTR during the first *postpartum* week with carbetocin could prevent the development of the pathological phenotype induced by PRS in both adulthood and aging. Here, I investigated whether carbetocin could abolish the development of cognitive dysfunction and abnormalities in glucose metabolism exhibited by PRS rats during ageing. This investigation is of particular relevance of the tight relationship between a defective glucose metabolism and the development of Alzheimer's disease and other types of dementia. To strengthen the relationship between abnormalities in glucose metabolism and age-related cognitive disorders I was also involved in a side project demonstrating that *O*-GlcNAcylation of brain proteins in general and tau protein in particular is reduced in the hippocampus of transgenic mice modeling Alzheimer's disease.

In addition to its role in mother-infant bonding, neurohypophyseal hormones such as oxytocin also play an important role in regulating the response to stress, by negatively controlling ACTH and corticosterone secretion (Windle et al., 1997; 2004; Neumann et al., 2000). In addition, the hippocampus is a key region in the negative control of HPA axis (Herman et al., 2005). Interestingly, most of the abnormalities induced by PRS are found in the ventral portion of the hippocampus (Zuena et al., 2008; Morley-Fletcher et al., 2011), a region encoding memories related to stress and emotions (Fanselow and Dong, 2010). Recent work from our group also demonstrated the existence of a causal relationship between reduced glutamate release in the ventral hippocampus and anxiety-like behavior in PRS rats (Marrocco et al., 2012). Because previous work of the laboratory showed that chronic antidepressant treatment was able to reverse most of the behavioral and neurochemicals alterations induced by PRS, in **CHAPTER TWO**, I wanted to assess whether carbetocin could correct synaptic impairments and associated stress-related behaviors in adult PRS rats. The effect of conventional antidepressants, fluoxetine and agomelatine on hippocampal glutamatergic synapses was also investigated in PRS rats.

Overall, this PhD thesis aimed at strengthening the view that changes in either prenatal and/or postnatal environment may cause permanent changes in the developmental trajectory of the offspring, thus shaping the vulnerability to CNS disorders later in life; in particular, maternal care variations are critical for the pathological programming induced by perinatal stress. Thus, consequences of the exposure to adverse events during sensitive time-windows are crucial feature in the vulnerability to mental and neurodegenerative disorders.

Results

CHAPTER ONE: Perinatal stress as a risk factor for age-related disorders

1. Pharmacological activation of oxytocin receptors in lactating dams prevents the long-term outcome of perinatal stress in the offspring

Large individual variability has been found in the vulnerability to develop neurological disorders. Early environmental factors such as exposure to stress during the perinatal period play an important role in the programming of diseases vulnerability. Indeed, early environmental triggers or stressors may have a permanent (but not irreversible) rather than a transient effect on the organism.

Study in animal models of stress-induced early-life programming allows the investigation of long-term consequences of perinatal stress on behavioral, neuroendocrine and neurochemical parameter all along the animal life (reviewed by Darnaudery and Maccari, 2008). In the previous section, we have seen that exposure to perinatal stress in rats programs the adult offspring to an increased anxiety- and depressive-like behavior (Vallée et al., 1996; Morley-Fletcher et al., 2011; Marrocco et al., 2012). During the early life, the transmission of the effects of perinatal stress from mother to fetus and then to the newborn, involves maternal factors such as increased levels of anxiety and plasmatic glucocorticoids associated with a decreased maternal care. Previous study of Prof. Maccari's group showed that the blockade of the mother's stress-induced glucocorticoids secretion by adrenalectomy during the gestational period, suppresses the prolonged stress-induced corticosteroid response in the adult offspring (Barbazanges et al., 1996). These results suggested that stress-induced increase in maternal glucocorticoids might be a mechanism by which PRS impairs the development of glucocorticoids response in the adult offspring (Fig. 21). In addition, in the early postnatal period, Maccari and colleagues (1995) showed that an early adoption of PRS rats by unstressed control mothers, a procedure that leads to increased maternal care, reversed the PRS effects on the HPA axis of adult rats and that stress during gestation induces long-lasting effects on emotional reactivity of the dam rat.

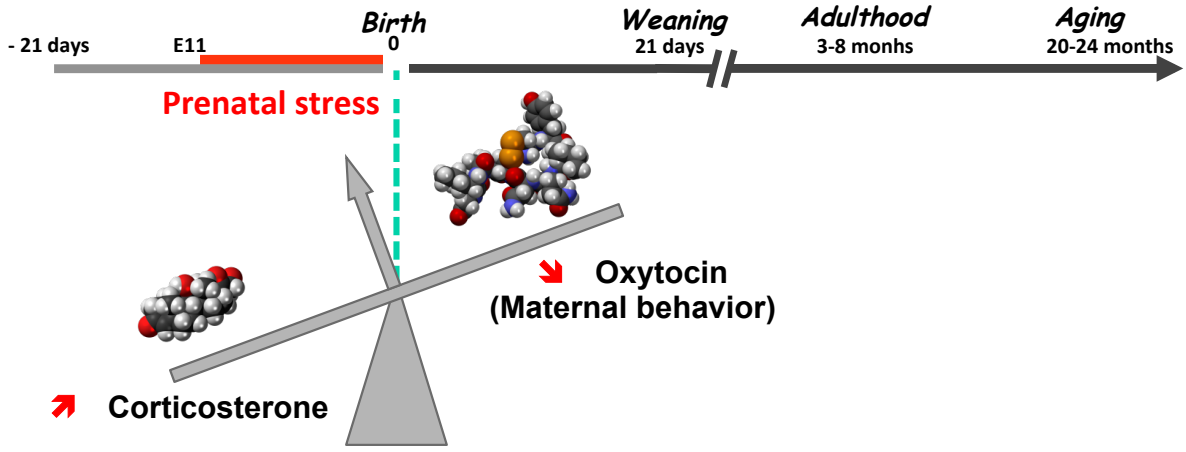

Figure 21: Prenatal restraint stress in rat alters the perinatal endocrine balance by increasing the prenatal glucocorticoids secretion and reducing the postnatal oxytocin production.

Recently, Murgatroyd and Spengler (2011) showed that early life stress in mice leads to epigenetic marking of the arginine vasopressin (AVP) gene underpinning sustained expression and increased hypothalamic-pituitary-adrenal axis activity. Glucocorticoids could also be involved on the programming of the fetal male hippocampal epigenome (Crudo et al., 2013). While glucocorticoids can be seen as a "negative" actors of programming, some other factors such as maternal behavior and oxytocin could be considered as "positive", because of its neuroprotective properties (reviewed by Vargas-Martínez et al., 2014) and its role in programming the reactivity to stress (Liu et al., 1997).

However, the involvement of the oxytocinergic system in the early phases of the pathological programming induced by gestational stress, and in the long-lasting expression of the PRS phenotype needs further investigations. In this chapter, I will investigate the role of glucocorticoids and oxytocin as the main hormonal actors of the programming induced by prenatal stress in rats by particularly focusing on the role of maternal behavior.

The therapeutic effect of an oxytocinergic analogue administration to lactating dams discloses a critical role for maternal care in the pathological phenotype induced by prenatal stress in the adult and aged offspring

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Abstract

The oxytocinergic system well known for its central effect on brain regions involved in the control of hypothalamic-pituitary-adrenal (HPA) axis activity and in the control of emotions and anxiety, also plays a critical role in mother/pup attachment and maternal behavior. Maternal care plays a crucial role in mediating environmental cues to ensure the survival and the adaptation of the offspring. Overexposure to adverse events early in life is known to induce pathological programming of the HPA axis in the offspring, thereby increasing the vulnerability to neuroendocrine disorders. We investigated whether the long-term consenquences induced by prenatal restraint stress (PRS) could result from changes in the early postnatal environement, e.g., maternal care.

Stressed and control unstressed mothers were treated with an oxytocin receptor agonist, carbetocin (1mg/kg, i.p.), during the first *postpartum* week. This treatment corrected the defect of maternal behavior and prevented the development of the pathological programming induced by early life stress in the offspring during life span. *Postpartum* carbetocin treatment in lactating mothers corrected the hyperreactivity of the HPA axis in response to stress in both adult and aged PRS offspring. In addition, carbetocin administration to lactating mothers persistently reduced the anxiety-like behavior of PRS rats and prevented the development of metabolic and spatial cognitive dysfunctions in aged PRS rats.

We conclude that the oxytocinergic system in the early life plays a protective role against the programming effect of adverse experiences. Thus, the oxytocinergic system could be considered as a novel potential therapeutic target for stress-related disorders.

Keywords: maternal behavior, carbetocin, oxytocin, early-life stress, anxiety.

Introduction

Mounting epidemiological studies have highlighted that exposure to adverse events during pregnancy can have detrimental long-term effects on the progeny. In mammals, maternal spontaneous behavior plays an important role in mediating the effects of the adverse environment to ensure growth survival and adaptation of the offspring. In rodents, the newly born pups are not fully mature and completely depend on their mother for feeding and care (Moriceau and Sulllivan, 2005).

The neuroendocrine system plays a crucial role in mother-pup attachment. In particular, oxytocin, known for its established role in parturition and lactation (Blanks and Thornton, 2003), is also critically involved in the onset of maternal behavior (Pedersen and Prange, 1979; Bosch and Neumann, 2012; Rich et al., 2014). Accordingly, pharmacological blockade of the oxytocinergic system impairs maternal behavior (Pedersen et al., 1985; van Leengoed et al., 1987; Pedersen and Boccia, 2003) and central infusion of oxytocin receptor antagonist eliminates natural variation in maternal care (Champagne et al., 2001).

During the first *postpartum* week natural variations in maternal behavior have been observed in lactating rodents (Champagne et al., 2003). Changes in the amount and quality of maternal care have been classified in terms of high and low licking/grooming (LG), and arched-back nursing (ABN) behaviors (Meaney, 2001). These variations in maternal care induce differences in the response to stress of the offspring, with the offspring reared by low LG-ABN mothers being more fearful and showing a hyperreactivity of the HPA axis to stress (Francis et al., 1999; Caldji et al., 2000). Thus, maternal care appears to be a major determinant for both neural development and the HPA axis response to stress of the offspring (Caldji et al., 1998; Liu et al., 1997; Champagne and Curley, 2009). A major question in studies of prenatal stress in rodents is whether the pathological consequences for the offspring result from events occurring during pregnancy, from changes in maternal care caused by gestational stress, or both. This question has been only partially addressed in rats exposed to prenatal stress, which represent a model of anxious/depressive disorders endowed with face, construct and pharmacological validity (Maccari et al., 1995; Morley-Fletcher et al., 2011; Mairesse et al., 2013; Marrocco et al., 2014). That changes in maternal care may be involed in the outcome of prenatal stress is suggested by the evidence that cross-fostering corrects the abnormality in the HPA axis response to stress caused by prenatal restraint stress (PRS) in the offspring (Maccari et al., 1995). An alternative strategy in addressing this question is the use of treatments delivered to the mothers during early *postpartum* period that enhance maternal behavior and cannot have

direct effects on pups during lactation. If effective, this treatment may also be proposed for human use in an attempt to restrain the development of CNS disorders that might be causally linked to early-life stress. Here we focused on the oxytocinergic system, treating lactating mothers exposed to gestational stress and their unstressed controls with carbetocin, a potent and selective oxytocin receptor agonist, which is brain permeant and shows a longer elimination half-life with respect to oxytocin (Dvorská et al., 1992). This drug is marketed for the treatment of *postpartum* bleeding in humans (Engstrøm et al., 1998). Carbetocin is a synthetic peptide that shows no oral bioavailability, and, therefore, cannot be transferred from lactating dams to pups throught the milk (Silcox et al., 1993). For this reason, this drug meets the requirement to be used as a pharmacological agent to disclose the role of maternal care in the developmental trajectory of PRS rats. Here, we examined the effect of carbetocin administration to lactating dams on the phenotype exhibited by PRS rats both in adult life and in the elderly. In aged rats we have extended the analysis to cognitive and metabolic functions that are influenced by prenatal stress.

Materials and Methods

Animals

Nulliparous female Sprague-Dawley rats (250-260 g, Charles River, France) were individually housed with a sexually experienced male for mating. A vaginal smear was performed in order to determine the beginning of the gestation. Presence of spermatozoa defined day 0 of gestation. Control dams were left undisturbed throughout gestation. Stressed dams were subjected to repeated episodes of restraint stress in a transparent cylinder (7.5 cm diameter, 19 cm long) under a bright light for 45 min three times daily from day 11 of pregnancy until delivery (Maccari et al., 1995). Three- or 16-22-month-old male offspring from litters containing 10-14 pups with an equivalent number of males and females were used. A maximum of one or two male pups were taken from each litter (Becker and Kowall, 1977; Chapman and Stern, 1979). Behavioral tests were performed between 9:00 A.M. and 1:00 P.M. All experiments were approved by the Institutional Animal Care and Use Committee according to the principles of laboratory animal care (European Communities Council Directive of 1986, 86/609/EEC) and following the Institute for Laboratory Animal Research ‘‘Guide for Care and Use of Laboratory Animals”.

Carbetocin treatment

Carbetocin (1mg/kg, SP080756, Polypeptide group, Strasbourg, France) or vehicle (saline) was administrated i.p. to lactating dams from postnatal day (PND) 1 to PND7. The dose and route of administration of carbetocin were selected on the basis of previous reports (Klenerova et al., 2009a,b; Mairesse et al., 2015).

Experimental protocol

Body weight was measured in dams during pregnancy, during the first postpartum week, and in the progeny at PND7, 3 months, and at different times during aging. In the adult and the aged progeny, behavioral, endocrinological and biochemical measurements were carried out in the temporal sequence indicated in fig. 1.

Analysis of maternal behavior

Control and stressed mothers were placed in standard transparent cages on a rack equipped with cameras. The video recording system included 28 small infrared cameras (CMTH with 1/4 Sony CCD, objective of 3.6mm) attached on a metal structure and placed about 12 cm distance

from the cage wall allowing the whole floor area detection (1 video camera per cage). A constant recording (24h/24h) was performed by two infrared LEDs pointed towards the ceiling to provide diffuse IR illumination in the room. Video signal were acquired on two 16 channels DVR encoding H.264 format (Avtech, AVC798ZA). The digital video signal was sent by IP to a computer for storage on a hard disk. Video Viewer Application® (version 0.1.8.4) drives the video recording and replay. From day 1 to day 5 after parturition, maternal active behavior (n=5 mothers per group) was analyzed offline (Noldus, The Observer, Wageningen, The Netherlands) for the 2 h following carbetocin or saline injection. Within each observation period, the behavior of each mother was scored every min (60 observations/h with 2 h of observation per 5 days, i.e. 121 observations/mother/day) for the following behaviors: feeding behavior (active arched back nursing, blanket posture in which the mother lays over the pups, or passive posture in which the mother is lying either on her back or side while the pups nurse), grooming, licking, carrying pups (Champagne et al., 2003). Data obtained correspond to the active presence of the mother on the nest expressed in percent respect to the total number of observations.

Anxiety-like behavior

Elevated Plus Maze - Both adult and old PRS or control offspring reared by mothers treated with saline or carbetocin were tested for anxiety-like behavior using a modified version of the elevated plus maze (EPM) (Pellow et al., 1985). For adult rats, the test was performed as described by Marrocco et al. (2012). For old rats, we used a custom-made EPM apparatus, which was 105 cm high, and consisted of 20 x 20 cm closed and open arms.

Spatial recognition

Y maze - Old rats (17 month-old) were tested using a 2-trial memory task between 9 AM and 3 PM, in a Y-maze as previously described (Dellu et al., 1992). The Y-maze consisted of 3 identical arms illuminated by a dim light (35 lux) and enclosed by 36 cm high side walls. Each arm was equipped with infrared beams and the Y-maze was linked to a computer. Numerous visual cues were placed on the wall of the testing room and were kept constant throughout behavioral testing. The floor of the maze was covered with dirty sawdust from the home cages of several animals, and was mixed between each passing in order to eliminate olfactory cues. The task consisted of 2 trials separated by a variable time interval (6 or 24h). During the first trial (acquisition phase), 1 arm of the Y-maze was closed and animals were placed in the center and allowed to explore the two other arms for 10 minutes. During the inter-trial interval (ITI, 6

or 24 hours), rats were housed in their home cages in a room different from the test room. During the second trial (test phase), the animals had free access to all 3 arms. The parameter evaluated was the time spent in the novel arm (the one closed during the first trial) during the first 3 minutes of the test phase. This parameter was expressed in percentages and was compared with the percentage of random chance exploration of the 3 arms (i.e., 33% for each arm). The animals were determined to have discriminated between the novel arm and the 2 familiar arms if the percentage of time spent in this arm was significantly superior to 33%. Memory performance was tested using 2 ITI (6 and 24 hours) separated by an interval of 1 week.

Morris Water Maze – The water maze test was used to investigate spatial learning in aged rodents (Morris, 1984). A plastic tank (2 m of diameters, 0.6 m height) was filled with water (22 ± 2 °C) up to 35 cm. Spatial cues were placed in the room and remained fixed throughout the experiment. Before test, animals were submitted to a two-day habituation phase, during which they were left for 1 min to explore the pool. During the test, animals were required to find a hidden platform (20 cm diameter) placed 3 cm below the water surface. The walls of the tank and the platform were black and indirect lightning was used in the room, enabling the platform to be hidden from animals' sight. All rats were submitted to 3 trials per day during 6 days and the starting positions changed over trials. Each trial began with the animal in the pool facing walls, and ended either after 90 s of swimming or when the animal found the platform. In either case, the rat was left on the platform for 20 s. Latency and distance to reach the platform were recorded using an automated system (Viewpoint, Lyon, France).

Social Interaction

Social interaction was assessed in 18 month-old control or PRS rats whose mother were treated with vehicle or carbetocin. The juvenile recognition ability was assessed using a procedure adapted from Engelmann et al. (1995). Rats were individually placed in transparent cages for 5 min for habituation. The challenged rat was first exposed to a male juvenile (2-month-old) for 5 min. Rats were exposed to a different juvenile 24h and one week later (three different juveniles were used in order to avoid habituation between sessions). Sessions were video-recorded and the time spent in interaction (sniffing, grooming, anogenital and play behavior) was measured by a trained observer using the Observer 20 (Noldus, Wageningen, The Netherlands).

Quantitative mRNA analysis

Rats were killed by decapitation, hippocampus was rapidly dissected and kept frozen at -80°C. RNA extraction was performed using the RNeasy Plus mini kit (Qiagen, France). RNA concentration in samples was determined using Nanodrop (ND-1000, Labtech, Germany), and quality verified by Rin (RNA Integrity Number; Bioanalyzer 2100, Agilent Technologies, France). Retrotranscription was performed with High-Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, France). Transcript levels were measured by real-time PCR using TaqMan assays (Applied Biosystems, France). TaqMan real-time PCR probes were obtained from Applied Biosystems: glucocorticoid receptor (GR, Nr3c1, Rn00561369_m1); mineralocorticoid receptor (MR, Nr3c2, Rn00565562 oxytocin/neurophysin 1 prepropeptide (oxt, Rn00564446_g1); oxytocin receptor (OxtR, Rn00563503_m1); arginine vasopressin (AVP; Rn00690189_g1). Transcript levels were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn001775763_g1) expression. Assay validity was assessed thanks to appropriate negative controls, in which cDNA was omitted. These negative controls were undetermined by software. Acquisition of data (threshold cycle, Ct) was performed by StepOnePlusTM software. 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 rats treated with saline) were calculated.

Corticosterone response to stress

Adult PRS and control rats, from mother treated with carbetocin at postpartum period were used for the analysis of the reactivity of the HPA axis in response to a mild stress. Animals were submitted to a 30 min novelty stress exposure during the first half of the light phase of the light/dark cycle (between 9:00 A.M. and 12:00 P.M.). Novelty stress consisted in placing the tested animal for 30 min. in a transparent cylindrical Plexiglas cage (30cm diameter, 50cm high) without sawdust and under a bright light (400 lux). Corticosterone levels were determined on plasma extracted from four blood samples (around 200 µl each) withdrawn from the tail vein for each animal. Samples were collected before stress (T0) and 30, 75 and 120 min afterward (Fig. 4).

In old animals plasma collection was performed after exposure to the last trial of the first session of Morris Water Maze (see below). Plasma corticosterone concentrations were determined using a commercial ELISA kit (DEV9922, Demeditec Diagnostics GmbH, Kiel, Germany). All standards, samples and controls were run in duplicate concurrently.

Measurements of oxytocin levels

Plasma oxytocin concentrations were determined in the plasma collected at the time of killing after 18 hours of fasting using a commercial ELISA kit (CSB-E12062p, Cusabio Biotech Co., France). All standards, samples and controls were run in duplicate concurrently.

Western Blot Analysis

Rats were killed by killed by decapitation. The ventral hippocampuswas rapidly dissected and immediately stored at -80°C. Tissues were homogenized at 4°C in a lysis buffer containing 320 mM sucrose, 5 mM HEPES, pH 7.4, 500 mM NaF, 10% SDS, 80 mM streptozotocine, and phosphatase and protease inhibitors. The bicinchoninic acid (BCA) assay was used for the determination of protein concentrations. Homogenized tissues were resuspended in Laemmli reducing buffer, and 20 µg of proteins were separated by electrophoresis on Criterion TGX 4-15% precast SDS-polyacrylamide gels (BioRad), and 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 mouse monoclonal anti-*O*-GlcNAc (1:2000 for high molecular weight proteins and 1:1000 for low molecular weight proteins, RL2; ThermoFisher Scientific; #MA1-072) and mouse monoclonal anti-β-actin (dilution 1:80.000, Sigma-Aldrich). All primary antibodies were incubated overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibody (purchased from GE Healthcare) was used (1:10 000) and incubated for 1 h at room temperature. Enhancing chemiluminescence detection system (Pierce ECL, Thermo Scientific) was used for detection on autoradiography films (GE Healthcare). Signals obtained β-Actin levels were quantified with the ImageJ software. Densitometric lane spetra profiles obtained for total *O*-GlcNAcylated proteins were generated by measuring the optical density (O.D.) of each line of pixel of a lane using a custom-built macro in ImageJ software.

Statistical analysis

Data were analyzed by two-way ANOVA or three-way ANOVA as indicated. The Fisher post-hoc test was used to isolate the differences. Correlations were analyzed using the Pearson's correlation analysis. Significance was set to $p < 0.05$.

Results

1. Postpartum carbetocin administration to dams corrects the early defect in maternal care caused by gestational stress

We first assessed the effect of gestational stress on body weight. During the last gestational week stressed dams showed a reduced body weight gain as compared to unstressed control dams (Fig. 2A). In contrast, no difference in body weight gain was found in the first postpartum week between stressed and unstressed mothers treated with saline. Interestingly, however, systemic carbetocin treatment to the dams from postpartum days 1 to 5 (1 mg/kg, daily, i.p.) increased body weight gain exclusively in stressed dams (Fig. 2B). We measured active maternal behavior (feeding behavior, grooming, licking, carrying pups) in stressed and unstressed dams receiving either saline or carbetocin during the first 5 postpartum days. In control dams treated with saline, maternal behavior decreased progressively from postpartum day 1 to 5. This decrease was sharper in stressed dams treated with saline, in which a highly significant reduction in active maternal behavior was found at postpartum days 2 and -3 with respect to either unstressed dams treated with saline (Fig. 2C). Treatment with carbetocin did not affect maternal behavior in unstressed dams, but abrogated the detrimental effect of gestational stress on maternal care (Fig. 2C).

2. Body weight of the offspring of dams exposed to gestational stress and treated with carbetocin during early postpartum period.

Offspring weight was monitored all life long. Pups reared by stressed dams treated with saline showed a slight reduction in body weight at postnatal day 7 (PND7) as compared to pups reared from unstressed control dams. Unexpectedly, carbetocin administration to both stressed and unstressed dams substantially reduced body weight of pups at PND7 in spite of the increased maternal care (Fig. 3A). Body weight did not differ among all groups of adult (3-month-old) offspring (Fig. 3B), but, interestingly, was lower in the aged offspring (17-22 months) of unstressed and stressed mothers treated with carbetocin (Fig. 3C).

3. Carbetocin administration to dams in the early postpartum period prevents the development of anxiety-like behavior and the heightened HPA response to stress induced by gestational stress in the adult progeny.

Adult offspring (3-month-old) of unstressed control and stressed dams treated with carbetocin or saline during the first postpartum week were tested for anxiety-like behavior in the elevated plus-maze, and plasma corticosterone levels in response to novelty stress. Expression of stress-related genes, i.e. NRC1, glucocorticoid receptor (GR) gene; NR3C2, mineralocorticoid receptor (MR) gene; OXT, oxytocin gene; OXTR, oxytocin receptor gene; AVP, vasopressin gene was measured in the hippocampus.

Adult PRS offspring of saline-treated mothers showed a reduced time spent in the open arm of the elevated plus-maze as compared to the offspring of unstressed mothers treated with saline, in agreement with previous findings (Zuena et al., 2008; Marrocco et al., 2012). Carbetocin administration to lactating mothers was able to prevent the increase in anxiety-like behavior in PRS animals, with no effect in control animals (Fig.4A). As expected, the corticosterone response to novelty stress was greater in PRS offspring of saline-treated mothers as compared to their respective controls. Carbetocin administration to lactating mothers normalized corticosterone response in adult PRS rats exposed to novelty stress (Fig. 4B).

Adult PRS rats reared by saline-treated mothers showed a significant reduction in MR mRNA levels, a trend to a reduction in OXT mRNA levels, and significant increases in OXTR and AVP mRNA levels in the hippocampus, as compared to adult control animals reared by saline-treated mothers. Treatment of lactating dams with carbetocin reversed all changes in the expression of stress-related genes caused by PRS (Fig. 4C). Again, carbetocin had no effect in the offspring of unstressed mothers (Fig. 4C).

We analyzed the correlation between behavioral and biochemical data of the adult progeny with the active maternal behavior at PND3. We found that the anxiety-like behavior in the elevated plus-maze showed a significant direct correlation with maternal behavior at PND 3. In contrast, AVP mRNA levels in the hippocampus were inversely related to maternal behavior at PND 3 (Fig. 4D). Corticosterone response to novelty stress and mRNA levels of other stress-related genes showed no or weak correlations with maternal behavior at PND 3 (not shown).

4. Carbetocin administration to dams in the early postpartum period prevents behavioral abnormalities induced by gestational stress in the aged progeny

The aged offspring (16 to 20-month-old) of mothers treated with saline or carbetocin were tested for anxiety-like behavior in the elevated plus-maze and spatial memory in the Y-maze and Morris Water Maze. The 16-month-old PRS offspring of saline-treated mothers showed an increased in anxiety-like behavior in the elevated plus-maze similar to that observed in adult progeny (Fig. 5A), indicating that the anxiety-prone programming caused by PRS was lasting across the animals' life span. The increase in anxiety-like behavior caused by PRS was prevented by carbetocin administration to lactating dams. Again, carbetocin had no effect on anxiety-like behavior in the control offspring of unstressed mothers (Fig. 5A). Anxiety-like behavior in the elevated plus-maze of the aged progeny was significantly correlated with maternal behavior at PND 3 (Fig. 5B).

The same groups of animals were tested for spatial learning in the Y maze after 1 month, social interaction after 2 months, and spatial learning in the Morris water maze after 4 months. The aged PRS offspring (17-month-old) of saline-treated mothers show a reduced performance in spatial recognition memory in the Y-maze with a 6 hours ITI, with a significantly reduced time spent in the novel arm (below the 33% cut-off chance level of random exploration). Carbetocin to lactating dams abolished the differences between control and PRS rats (Fig. 6A). After an ITI of 24 hours recognition memory fell in every group, with no difference being observed among groups (data not shown). The spatial memory performance in the Y maze (6 h ITI) of the aged progeny was significantly correlated with maternal behavior at PND 3 ($r = 0.35$, $p = 0.027$; data not shown). At the end of the Y-maze test, samples of peripheral blood were collected for measurements of glucose levels. PRS rats reared by mothers treated with saline showed an increase in blood glucose levels, which was prevented by carbetocin (Fig. 6B). Changes in post-Y maze glucose levels were inversely correlated with maternal behavior at PND3 (Fig. 6C).

Social interaction toward a juvenile was assessed in the 18-month-old offspring. At day 1, 2 and 7 the aged PRS offspring of saline-treated mothers showed a reduced time spent in social interaction as compared to the respective control animals. Postpartum carbetocin treatment to dams abolished the differences between control and PRS rats in all days of testing (Fig. 6D). We found that social interaction in the aged progeny was positively correlated with maternal behavior at PND 3 at day 1 (Fig. 6E) and 7 ($r = 0.39$, $p = 0.029$; data not shown).

Spatial learning was assessed with the Morris Water Maze in 20-month-old rats. In all groups, the latency to reach the hidden platform progressively decreased over the consecutive days of testing (Fig. 7A). The distance covered to reach the platform also decreased over the days of testing (not shown). When values were expressed as a percentage of day 1, the latency to reach the platform was significantly increased at day 4, 5 and 6 of testing in aged PRS offspring (20-month-old) reared by saline-treated mothers (see inset of Fig. 7A). Carbetocin administration to lactating dams abolished the effect of PRS on spatial memory in the water maze in the aged offspring without affecting spatial memory in control aged rats (Fig. 7A). Measurements of corticosterone levels in the peripheral blood collected after the last trial in the Morris water maze (a stressful condition) showed that the HPA axis was hyper-reactive in the aged PRS offspring, and this was reversed by carbetocin administration to lactating dams (Fig. 7B). Post-water maze corticosterone levels showed an inverse correlation with maternal behavior at PND3 (Fig. 7C).

5. Carbetocin administration to dams in the early postpartum period prevents changes in fasting plasma glucose, corticosterone and oxytocin levels induced by gestational stress in the aged progeny.

Two months after the behavioral testing in the Morris water maze, all animals were killed after 18 hours of fasting, and the blood was collected for measurements of glucose, corticosterone, and oxytocin levels. Aged PRS rats reared by mothers treated with saline showed a trend to a reduction in glucose levels, a significant increase in corticosterone levels, and a significant reduction in oxytocin levels. All these changes were prevented by *postpartum* carbetocin treatment (Fig. 8A-C). Corticosterone levels were inversely correlated with maternal behavior at PND3 ($r = -0.41$, $p = 0.018$; data not shown). A positive correlation was also found between plasma oxytocin levels and maternal behavior ($r = -0.38$, $p = 0.031$; data not shown). No correlation was found between fasting glucose and maternal behavior (not shown).

6. Carbetocin administration to dams in the early postpartum period enhance the* O-*GlcNAcylation of high-range molecular weight proteins.

We measured the levels of the *O*-GlcNAcylated proteins obtained from total homogenates from the ventral hippocampus (Fig. 9). The optical density (O.D.) of all detectable bands obtained in total protein extracts from the ventral hippocampus of aged control and PRS rats reared by saline- or carbetocin-treated mothers show a ladder of *O*-GlcNAcylated proteins. No significant differences were found in the spectrum of overall protein *O*-GlcNAcylated proteins of the

ventral hippocampus. Remarkably, in protein around 170kDa aged PRS reared by saline-treated mother exhibit a trend to reduction in *O*-GlcNAc levels that is corrected when PRS rats are reared by carbetocin-treated mothers. A similar pattern is found in *O*-GlcNAcylated protein of 130kDa but did not reach statistical significance.

Discussion

Here, we have shown for the first time a reduction of pup-directed maternal behavior in our model of prenatal restraint stress in Sprague-Dawley rats. This is consistent with data obtained with different paradigms of prenatal stress in different strains of rats (Moore and Power, 1986; Champagne and Meaney, 2006). Of note, maternal care was reduced during the first four *postpartum* days, which are critical for shaping the development of the pup under the influence of mother-pup interaction. This raises a critical question, which has been consistently addressed since the early times of development of our PRS model, of wheter the prenatal environment (e.g., hormonal or metabolic signals transferred from the stressed mother to the fetus) or rather the defect in maternal care is critical for the pathological phenotype of the PRS offspring. The use of drugs that may be effective in correcting maternal behavior, but can be transferred to the pup through the milk, may generate data that are difficult to explain or might even be paradoxical. For example, treatment to dams exposed to gestational stress with the SSRI citalopram did not prevent anxiety- and depressive-like behavior in the offspring (Zohar et al., 2015). Thus, targeting the ocytocinergic system could represent an efficient strategy.

Our finding that early *postpartum* carbetocin administration to the mother corrects maternal behavior and prevents all pathological outcomes of prenatal stress throughout the entire lifespan of the offspring strongly suggests that gestational stress influences the development of the offspring postnatally, by causing an early defect in maternal care. This hypothesis is in line with the evidence that cross-fostering corrects the hyperactivity of the HPA axis induced by PRS in the adult offspring (Maccari et al., 1995).

The increased maternal care induced by early postnatal carbetocin administration in mothers that had been exposed to gestational stress might be a consequence of the anti-stress and anxiolytic effects resulting from activation of oxytocin receptors in the CNS (Windle et al., 1997; 2004). This might explain why carbetocin administration to stressed mothers increased body weight in the postpartum period, in spite of the documented anorexiogenic activity of oxytocin (Blevins and Ho, 2013). We were surprised to observe a reduced weight gain in pups reared by mothers treated with carbetocin regardless of whether dams had been stressed or not during gestation. This finding is difficult to explain if we assume that carbetocin should have increased lactation by stimulating oxytocin receptors in mammary glands (Nickerson et al., 1954). Perhaps, the continuous stimulation of oxytocin receptors resulting from the long half-life of carbetocin might have caused a paradoxical reduction of nutritional supply for the pups owing to milk secretion uncoupled from suckling. This hypothesis remains to be demonstrated.

Epidemiological studies have consistently shown that a low body weight at birth is a predictor of poor linear growth and enhances vulnerability to metabolic and cardiovascular disorders in the adulthood (Barker et al., 1993; Barker, 2004; Gluckman et al., 2005). Clearly, this was not the case in our rats reared by carbetocin-treated mothers, which fully recovered body weight in the adulthood, and even showed a reduced body weight during aging (which is expected to be protective against metabolic and cardiovascular disorders).

Correction of maternal care by carbetocin in stressed dams had a dramatic impact on the PRS phenotype across the entire life span of the offspring, by preventing all pathological manifestations we have seen in both adult (Darnaudéry and Maccari, 2008) and aged offspring (Vallée et al., 1999; Lesage et al., 2004). In aged PRS rats, these manifestations included cognitive dysfunction and a reduced protein *O*-GlcNAcylation in the hippocampus. This raises the attractive possibility that pharmacological activation of oxytocin receptors during the early *postpartum* period in mothers exposed to gestational stress might protect children not only during the critical period of their development, but all life long.

One point that we have only partially addressed is whether gestational stress causes a defect in the oxytocinergic system in lactating mothers and/or in the offspring. Gestational stress has been shown to reduce oxytocin mRNA levels in the hypothalamus (Hillerer et al., 2011), and amygdala (Murgatroyd et al., 2015) in dams. In addition, mothers previously selected for high levels of maternal care showed a reduced oxytocin receptor binding in the amygdala, septum and hypothalamus in response to gestational stress (Champagne and Meaney, 2006). In the adult PRS offspring we observed a reduced oxytocin mRNA levels associated with a compensatory increase in oxytocin receptor mRNA levels in the hippocampus. When exactly these modifications begin during postnatal development is unknown. However, they were directly related to the reduction in maternal care because they were corrected by carbetocin treatment to the mother. A critical role for maternal care in the development of the central oxytocinergic system in the offspring is also suggested by the evidence that cross-fostering has prevents the reduction in central oxytocinergic tonus caused by prenatal stress (de Souza et al., 2013). Thus, the activity of the oxytocinergic system in lactating mothers might be permissive to the development of the hippocampal oxytocinergic system in the offspring, explaining why this treatment could reverse the pathological phenotype caused by early life stress. In addition, the reduced availability of oxytocin and the resulting compensatory hyperexpression of oxytocin receptors in the hippocampus help to explain why carbetocin treatment to adult PRS

rats was highly effective in correcting the pathological phenotype of these animals (Mairesse et al., 2015).

The evidence that the improvement in maternal behavior caused by carbetocin treatment to the mothers was able to prevent not only the anxious phenotype in the adult PRS rats, but also the defects of spatial learning and social interaction, and all metabolic abnormalities of aged rats suggests that all these manifestations are components of an unique developmental programming, which is critically controlled by maternal behavior. Thus, maternal care may act as a master switch of all possible developmental trajectories in the offspring. One question that is particularly relevant to pathophysiology of cognitive disorders in the elderly is how impairment in glucose metabolism contributes to cellular and synaptic dysfuntion leading to dementia. Studies carried out in models of Alzheimer's disease (AD) strongly suggest that a perturbance of neuronal glucose uptake with the resulting impairment of glucose-dependent intracellular processes lies at the core of the disorders (Giuffrida et al., 2012; 2015). Among the possible consequences of a defective glucose metabolism there is a reduction of *O*-GlcNAcylation of intracellular proteins, including proteins, such as tau, which are heavily implicated in the pathophysiology of AD and frontotemporal dementia. We have recently found that transgenic mice modeling AD show a reduced *O*-GlcNAcylation of tau, which is possibly linked to tau hyperphosphorylation and formation of neurofibrillary tangles (Gatta et al., 2016). This suggests that a reduced protein *O*-GlcNAcylation we have found in aged rats might predispose these animals to a dementia-like pathology. The study of tau protein in these animals will be fundamental to address this specific issue. The mechanism by which *O*-GlcNAcylation is reduced in PRS rats remains to be determined. One possibility is that a lowered glucose uptake in neurons or other brain cells caused by central insulin resistance or other mechanisms reduces the availability of the major metabolic precursor of UDP-GlcNAc. Another possibility is that the reduced glutamate release in in the hippocampus of PRS rats (Marrocco et al., 2012; Mairesse et al., 2015) could result from less glutamine (another metabolic precursor of UDP-GlcNAc) that is synthesized in astrocytes and/or transferred from astrocytes to neurons. This could contribute to the reduced *O*-GlcNAcylation levels we have found in the ventral hippocampus of PRS rats (see Fig. 9). These two hypotheses are not mutually exclusive.

Whatever the mechanism, our data raise the interesting possibility that treatments that improve maternal care, including oxytocin receptor agonists might eliminate a potential risk factor for

the development of late cognitive disorders by counteracting the detrimental effect of early life stress on mother-pup interaction.

In conclusion, our findings provide new insights into the complex and multifaceted interaction between oxytocin and stress indicating that this interaction is involved in both the development and late expression of the pathological programming induced by early life stress. In addition, data support the use of oxytocin receptor agonists in the treatment of stress-related disorders, and raise the interesting possibility that this drug administered to lactating mothers restrains the pathological consequences of early life stress throughout the entire lifespan of the offspring.

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Conflict of interest

The Authors declare no conflict of interest.

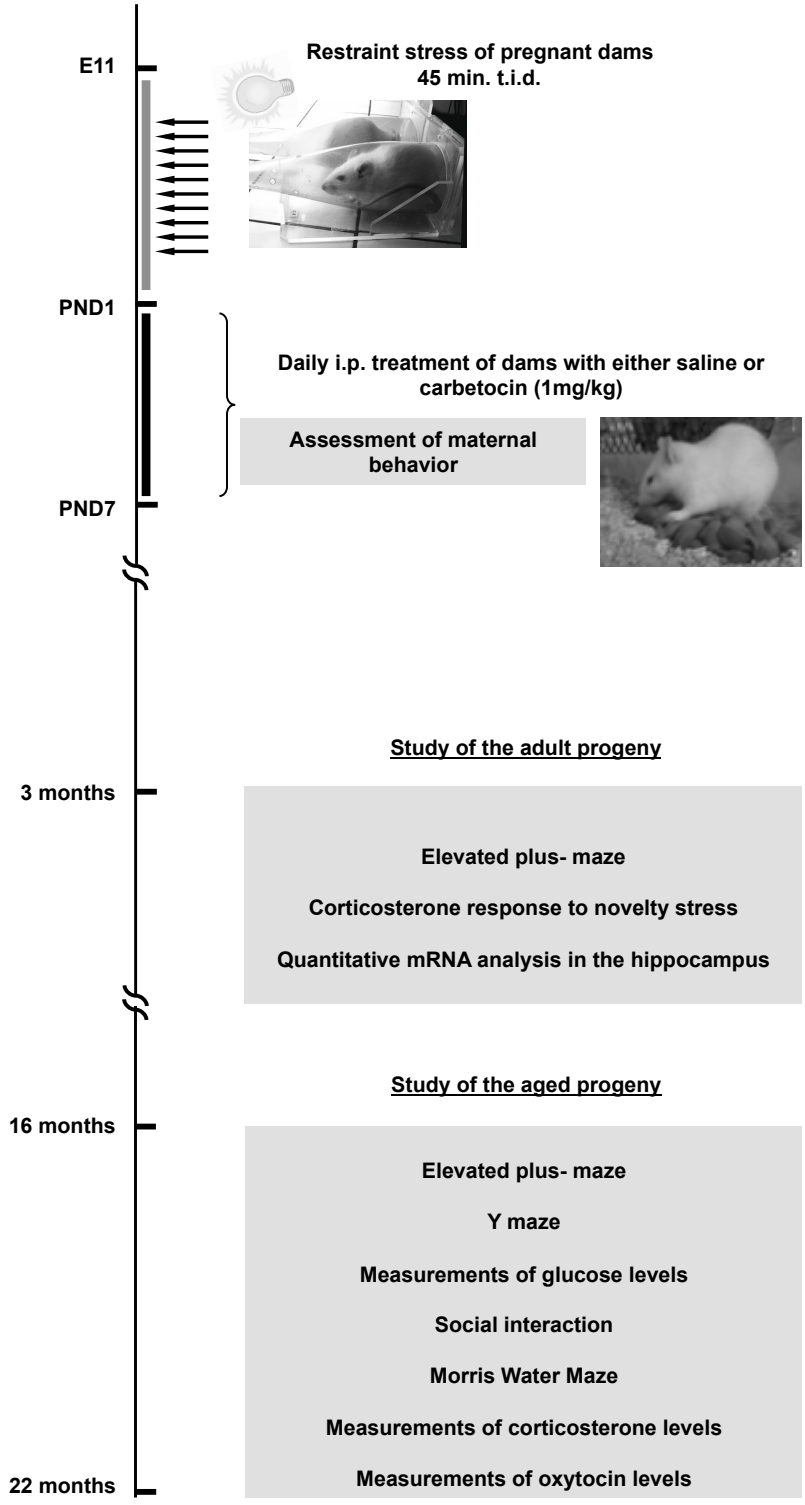

Fig. 1 – Time-line of the experimental procedures.
Body weight was measured in dams during pregnancy, during the first postpartum week, and in the progeny at PND7, 3 months, and at different times during aging. In the adult and the aged progeny, behavioral, endocrinological and biochemical measurements were carried out in the temporal sequence reported in the grey boxes. Abbreviations: t.i.d. = three times per day (ter in die); E11 = embryonic day 11; PND1/7 = postnatal day 1/7.

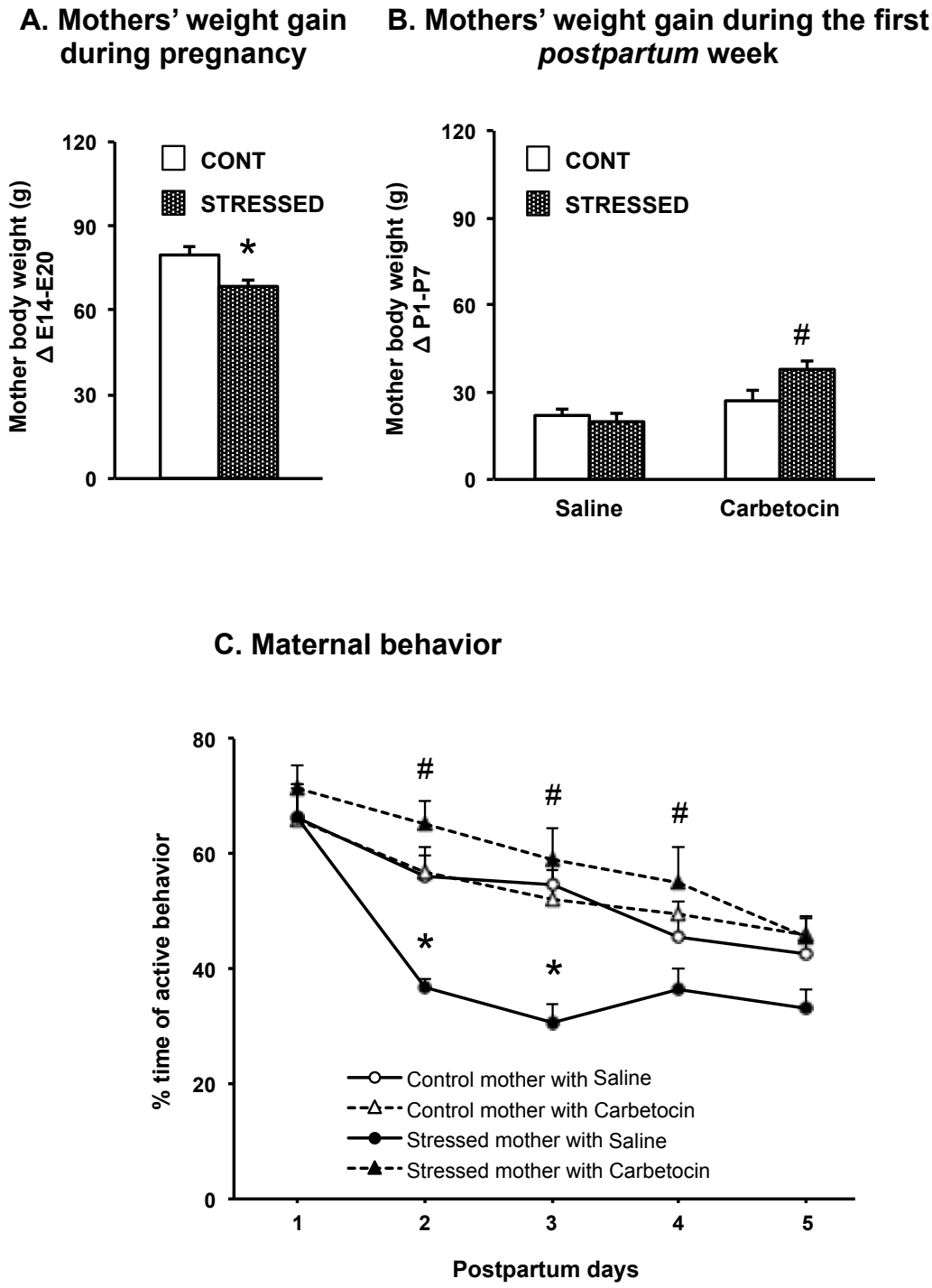

Fig. 2 – Postpartum carbetocin administration to dams corrects the early defect in maternal care caused by gestational stress.

Mothers' weight gain during the last gestational week and during the first postpartum week is shown in A and B, respectively. Values are means ± S.E.M. (n = 11 per group in A and 5 per group in B). * $p < 0.05$ vs. the unstressed control group (C) (Student's t test; $t_{1\text{-}20} = 2.57$); # $p < 0.05$ vs. the respective group treated with saline (Two-Way ANOVA + Fisher's LSD; treatment $F_{1,16} = 15.76$). Maternal behavior in the first five postpartum days is shown in C, where values are means ± S.E.M. (n = 5 per group). $p < 0.05$ vs. the unstressed control group (*) or vs. the groups of unstressed mothers treated with saline (#) at the respective time points.

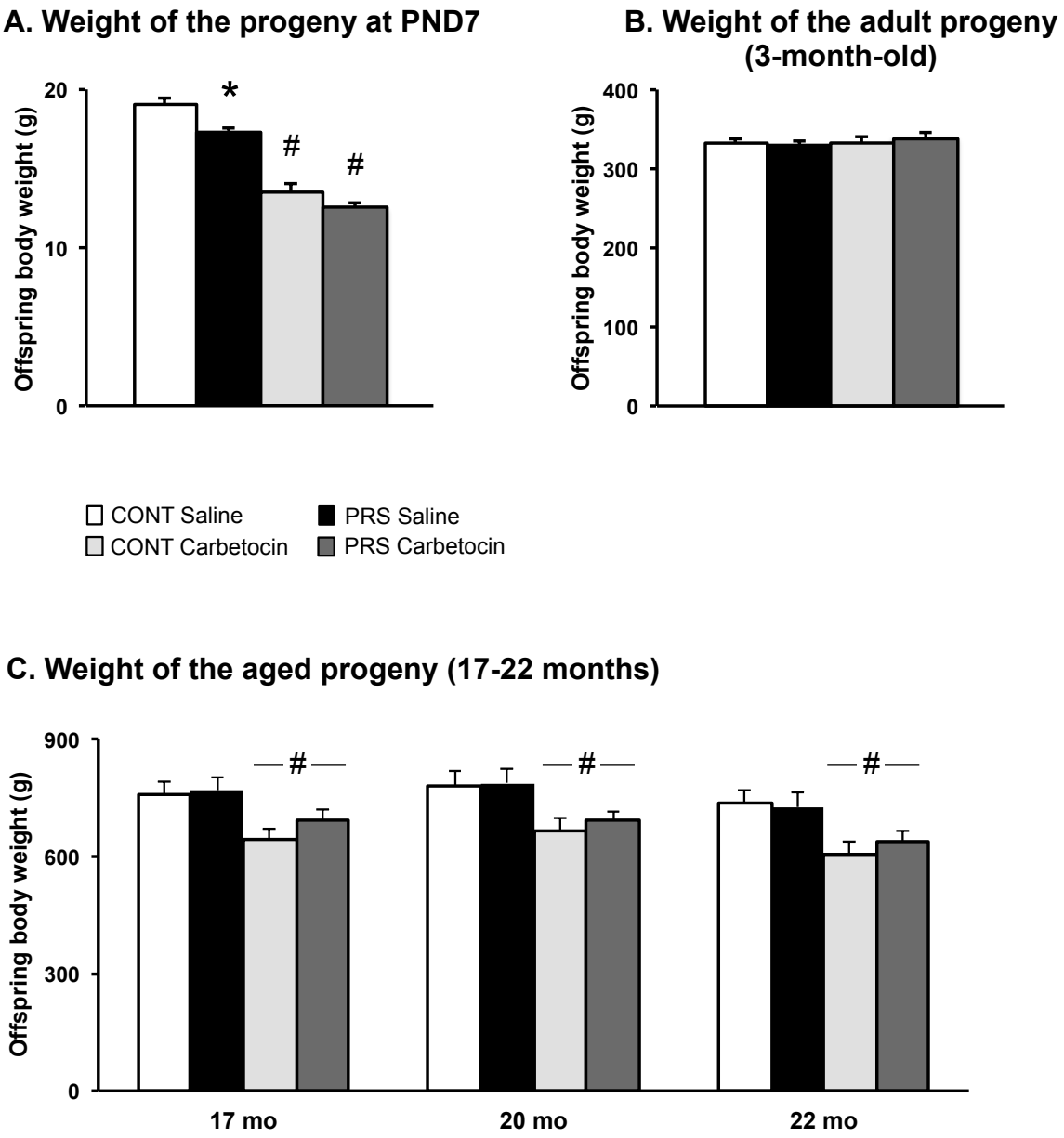

Fig. 3 – Body weight of the offspring of dams exposed to gestational stress and treated with carbetocin during early postpartum period.

Body weight of the progeny at PND7 (A), 3 months (B), and 17-22 months (mo) of age (C). Values are means ± S.E.M of 5 animals per group in A and B, and 8 animals per group in C. $p < 0.05$ *vs.* the respective control groups (offspring of unstressed dams) (*) or *vs*. the respective controls (CONT) or PRS saline groups (#) (Two-Way ANOVA + Fisher's LSD; A: group $F_{1,16} = 6.12$; treatment $F_{1,16} = 87,58$; C: treatment $F_{1,28} = 9.19$, 10.97, and 12.02, at 17, 20, and 22 months, respetively).

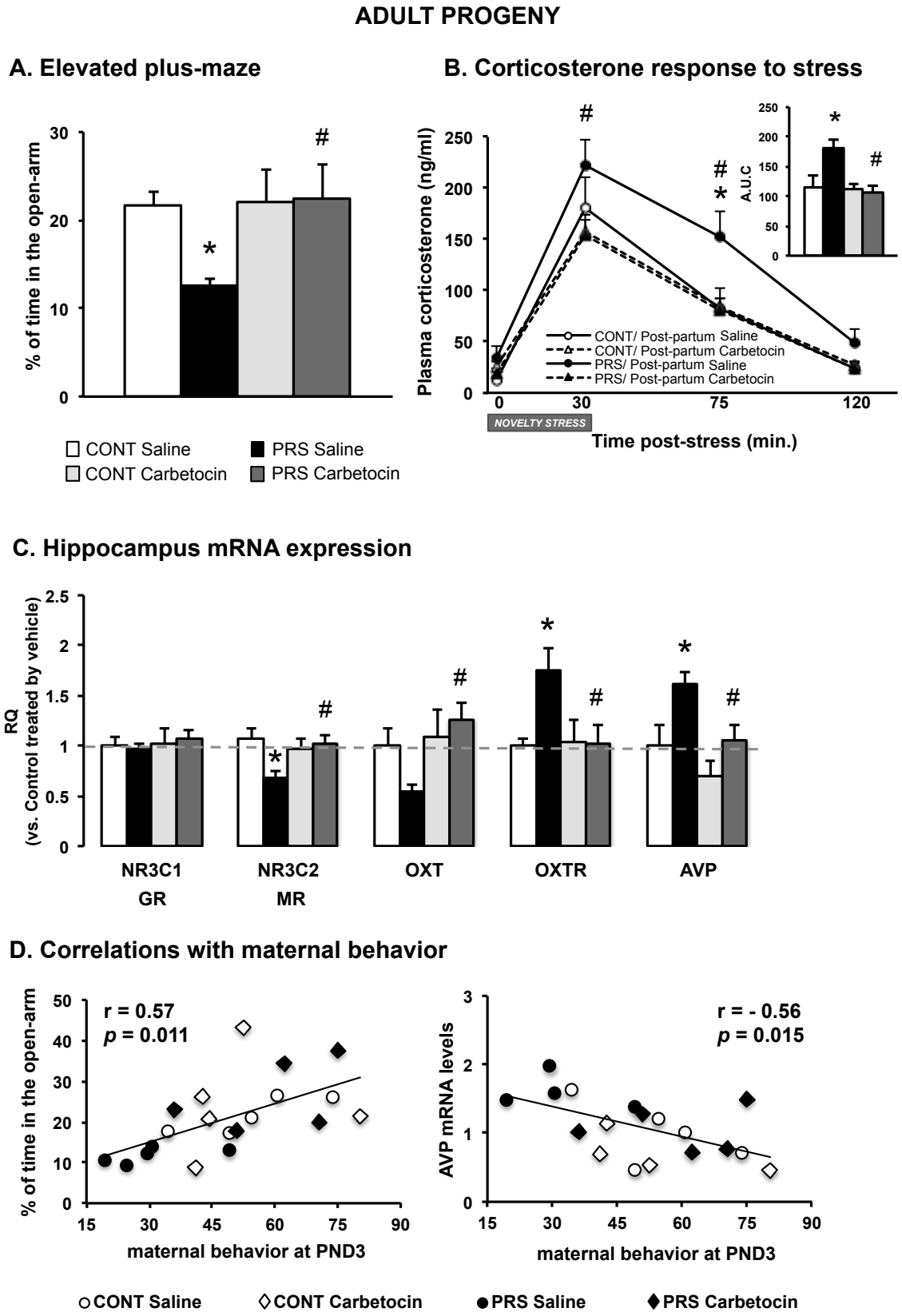

Fig. 4 – Carbetocin administration to dams in the early postpartum period prevents the development of anxiety-like behavior and the heightened HPA response to stress induced by gestational stress in the adult progeny.

Anxiety-like behavior in the adult (3-month-old) offspring of stressed and unstressed dams treated with saline or carbetocin is shown in A, where values are means ± S.E.M of 5 animals per group. $p < 0.05$ *vs.* the unstressed control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; group $F_{1,25} = 6.32$, group x treatment $F_{1,25} = 5.99$).
Corticosterone response to novelty stress is shown in B. In the inset: A.U.C. values. Values are means ± S.E.M of 5 animals per group. $p < 0.05$ *vs.* the unstressed control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (main graph: Three-Way ANOVA for repeated measures + Fisher's LSD; treatment, $F_{1,16} = 6.51$, time effect, $F_{3,48} = 100$, group x treatment effect, $F_{1,16} = 6.11$; inset: Two-Way ANOVA + Fisher's LSD; treatment $F_{1,16} = 6.08$, group x treatment effect, $F_{1,16} = 5.77$).
Quantitative mRNA analyses of stress-related genes in the hippocampus is shown in C, where values are means ± S.E.M of 4-5 animals per group. $p < 0.05$ *vs.* the unstressed control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; mineralocorticoid receptor (MR): group x treatment $F_{1,15} = 6.29$; oxytocin (OXT): treatment $F_{1,12} = 4.54$; oxytocin receptor (OXTR) group x treatment $F_{1,16} = 4.36$; vasopressin (AVP) group $F_{1,14} = 8.24$, treatment $F_{1,14} = 6.44$. Abbreviations: NRC1, glucocorticoid receptor (GR) gene (Mean cycle threshold (Ct) of control offspring of saline-treated unstressed dams = 22.8); NR3C2, mineralocorticoid receptor (MR) gene (Ct = 21.5); OXT, oxytocin gene (Ct=32.9); OXTR, oxytocin receptor gene (Ct = 27.3); AVP, vasopressin gene (Ct = 28.9).
Correlation analyses between maternal behavior at PND3 and anxiety-like behavior in the elevated plus-maze or AVP mRNA levels in the hippocampus are shown in D.

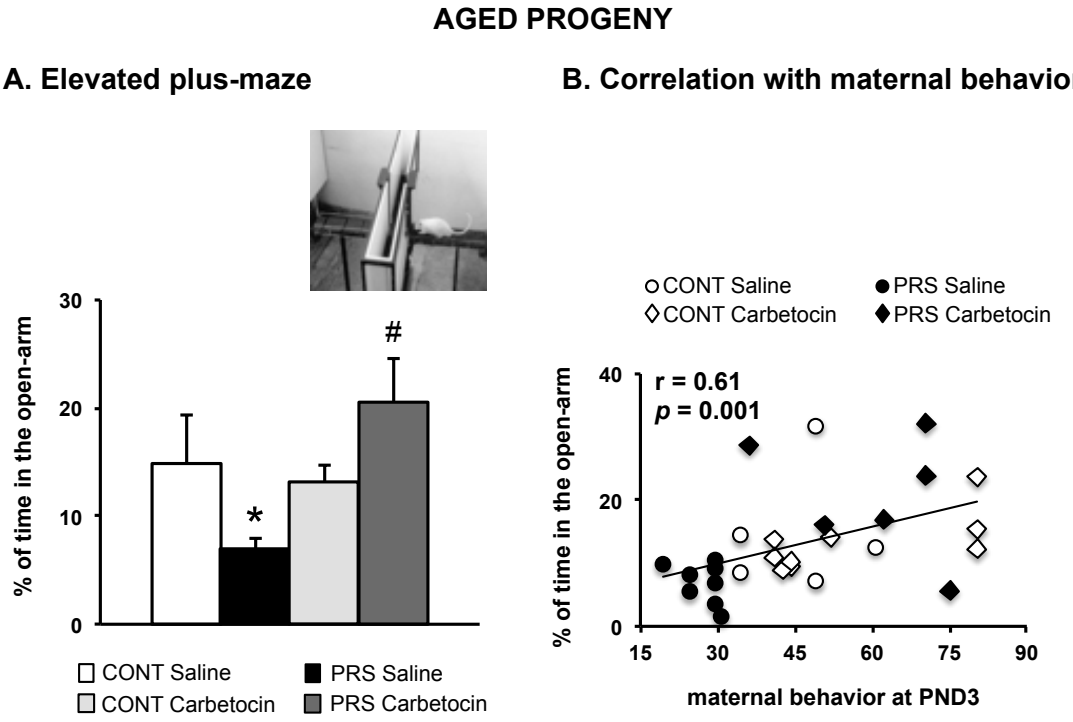

Fig. 5 – Carbetocin treatment to lactating dams prevents the development of anxiety-like behavior in the perinatally stressed aged progeny.

Behavioral tests were performed in the same groups of aged animals in spite of the variability of the n values in the different tests. Data of the elevated plus-maze in 16-month-old rats are shown in A, where values are means ± S.E.M of 5-8 animals per group. $p < 0.05$ *vs.* the unstressed control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; treatment $F_{1,24} = 5.2$, group x treatment $F_{1,24} = 8.57$). Correlation analysis between elevated plus-maze data and maternal behavior at PND3 is shown in B.

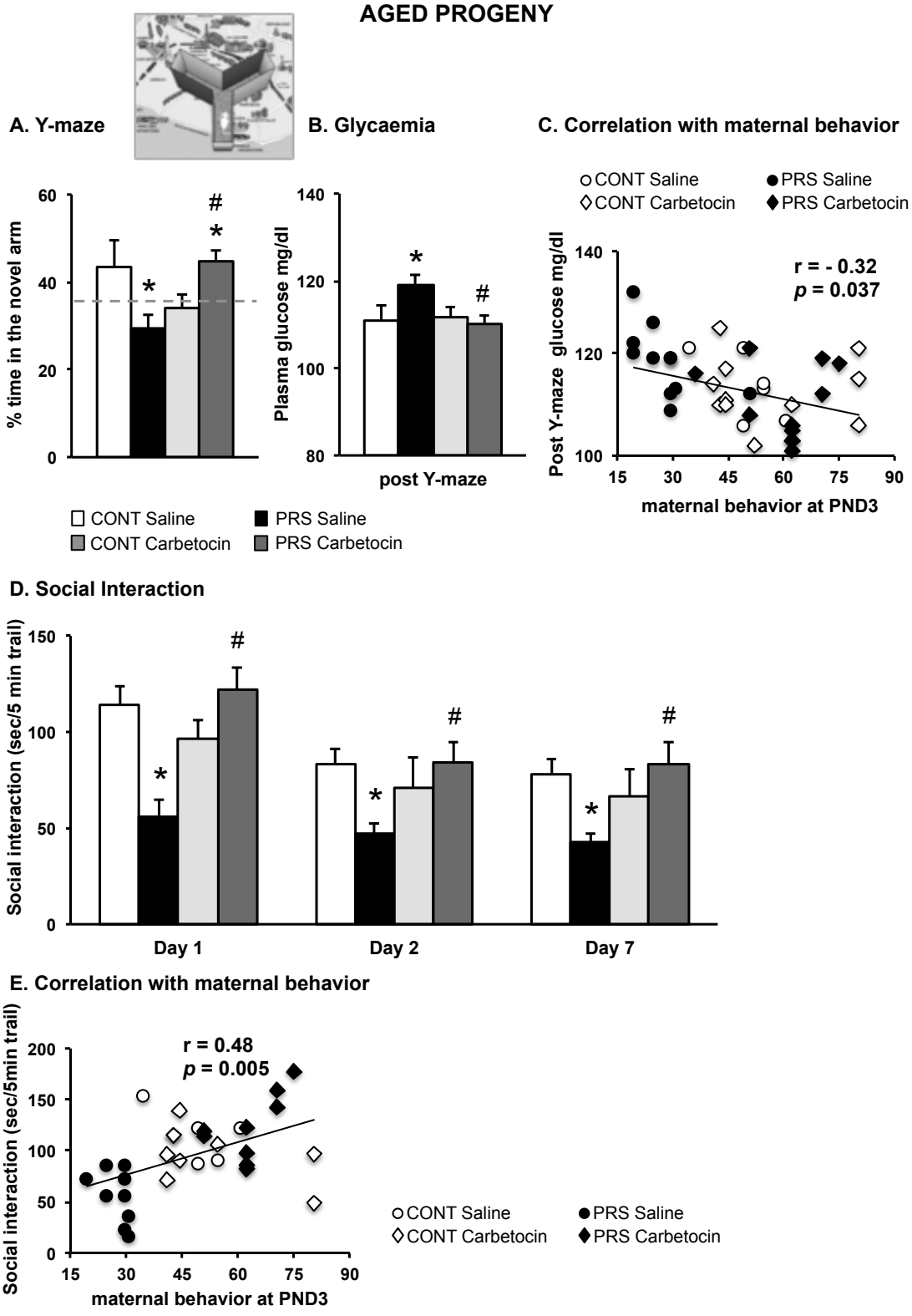

Fig. 6 - Carbetocin administration to dams in the early postpartum period prevents behavioral abnormalities induced by gestational stress in the aged progeny.

Y maze data in 17-month-old rats are shown in A, where values are means ± S.E.M of 5-14 animals per group. $p < 0.05$ *vs.* the respective unstressed control groups (CONT Saline or CONT Carbetocin) (*) or *vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; group x treatment $F_{1,36} = 11.67$).

Plasma glucose levels were measured immediately after the performance on the Y maze in 16-month-old rats (B). Values are means ± S.E.M of 7-13 animals per group. $p < 0.05$ *vs.* the respective control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; group x treatment $F_{1,37} = 4.03$).

Correlation analysis between Y maze data and maternal behavior at PND3 is shown in C.

Data of social interaction at days 1, 2 and 7 in 18-month-old rats are shown in D, where values are means ± S.E.M of 6-9 animals per group. $p < 0.05$ *vs.* the respective control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Three-Way ANOVA for repeated measures + Fisher's LSD; treatment $F_{1,28} = 4.35$, group x treatment $F_{1,28} = 13.67$, time (days) $F_{2,56} = 16.33$). Correlation between total interaction values (sum of values of days 1, 2 and 3) and maternal behavior at PND3 is shown in E.

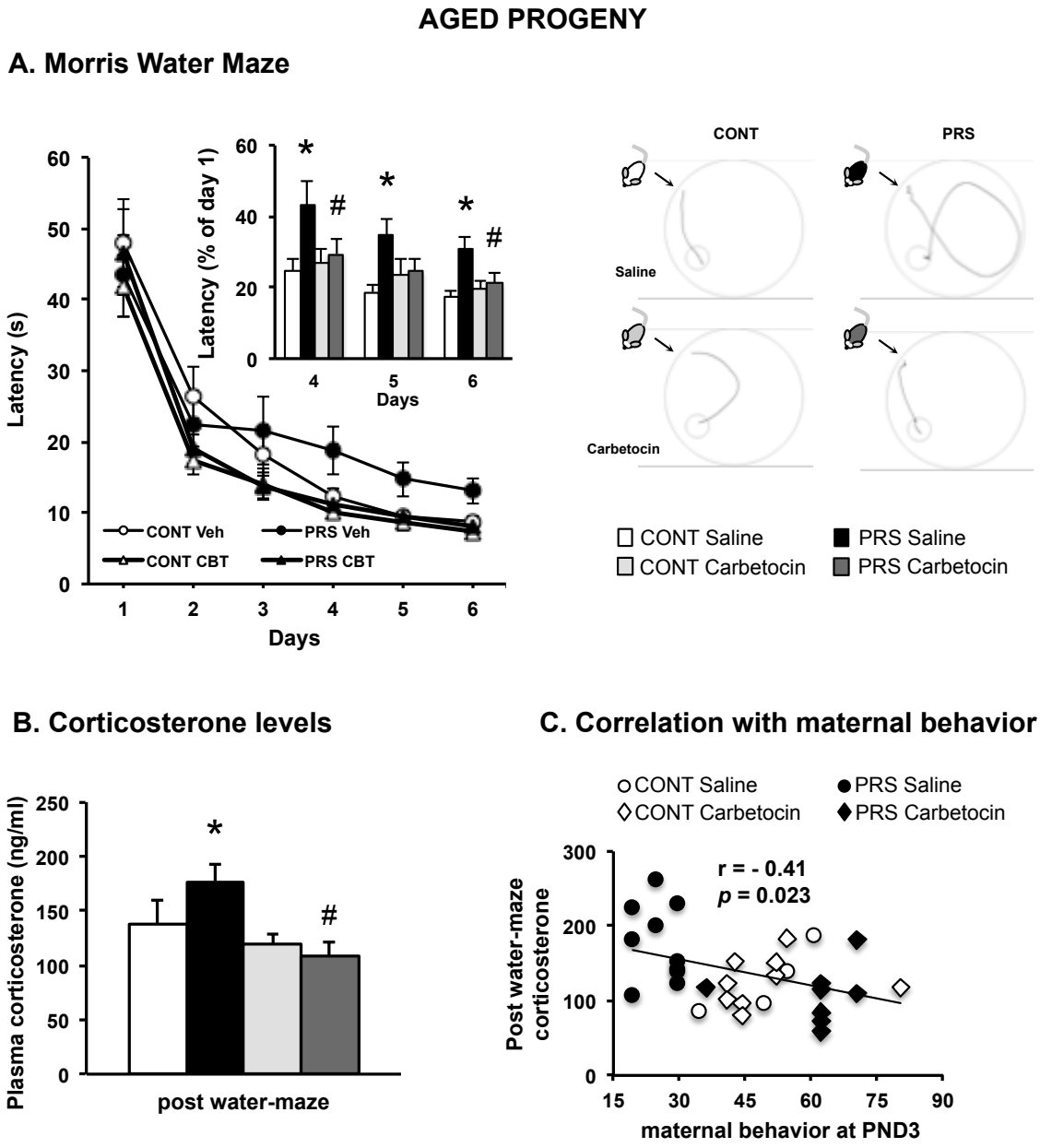

Fig. 7 - Carbetocin administration to dams in the early postpartum period prevents the defect in spatial memory performances induced by gestational stress in the aged progeny.

Data of Morris Water Maze (latency to reach the platform) in 20-month-old rats are shown in A, where values are means ± S.E.M of 6-13 animals per group. $p < 0.05$ *vs.* the respective control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Three-Way ANOVA for repeated measures + Fisher's LSD; latency (s): treatment $F_{1,37} = 4.69$, time $F_{5,185} = 106.57$, time x group x treatment $F_{5,185} = 2.32$; Two-Way ANOVA + Fisher's LSD; latency (% day 1) day 4: group x treatment $F_{1,37} = 4.19$; -day 5: group $F_{1,37} = 4.28$; -day 6: group $F_{1,37} = 8.98$, group x treatment $F_{1,37} = 5.69$). Representative trajectories obtained at the 4th day of test are presented in the right panel.

Plasma corticosterone levels were measured immediately after the last trial of the water maze test in 20-month-old rats (B). Values are means ± S.E.M of 5-10 animals per group. $p < 0.05$ *vs.* the respective control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; treatment $F_{1,27} = 7.99$).

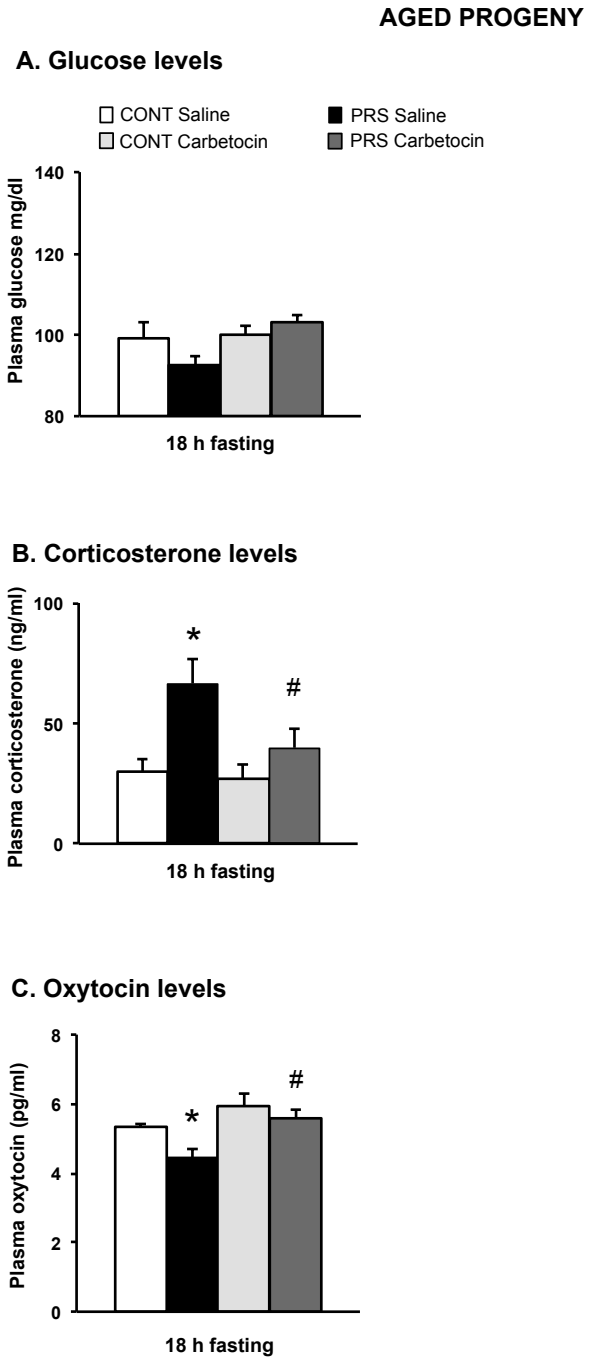

Fig. 8 – Carbetocin administration to dams in the early postpartum period prevents changes in on plasma glucose, corticosterone and oxytocin levels induced by gestational stress in the aged progeny.

All measures were performed on plasma obtained after 18 hours of fasting at the time of killing in 22-month-old rats.

In A, plasma glucose levels. Values are means ± S.E.M of 5-10 animals per group.

In B, plasma corticosterone levels. Values are means ± S.E.M of 6-10 animals per group. $p < 0.05$ *vs.* the respective control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; basal: group $F_{1,29} = 8.51$).

In C, plasma oxytocin levels. Values are means ± S.E.M of 5-10 animals per group. $p < 0.05$ *vs.* the respective control group (CONT Saline) (*) or *vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; treatment $F_{1,29} = 7.34$).

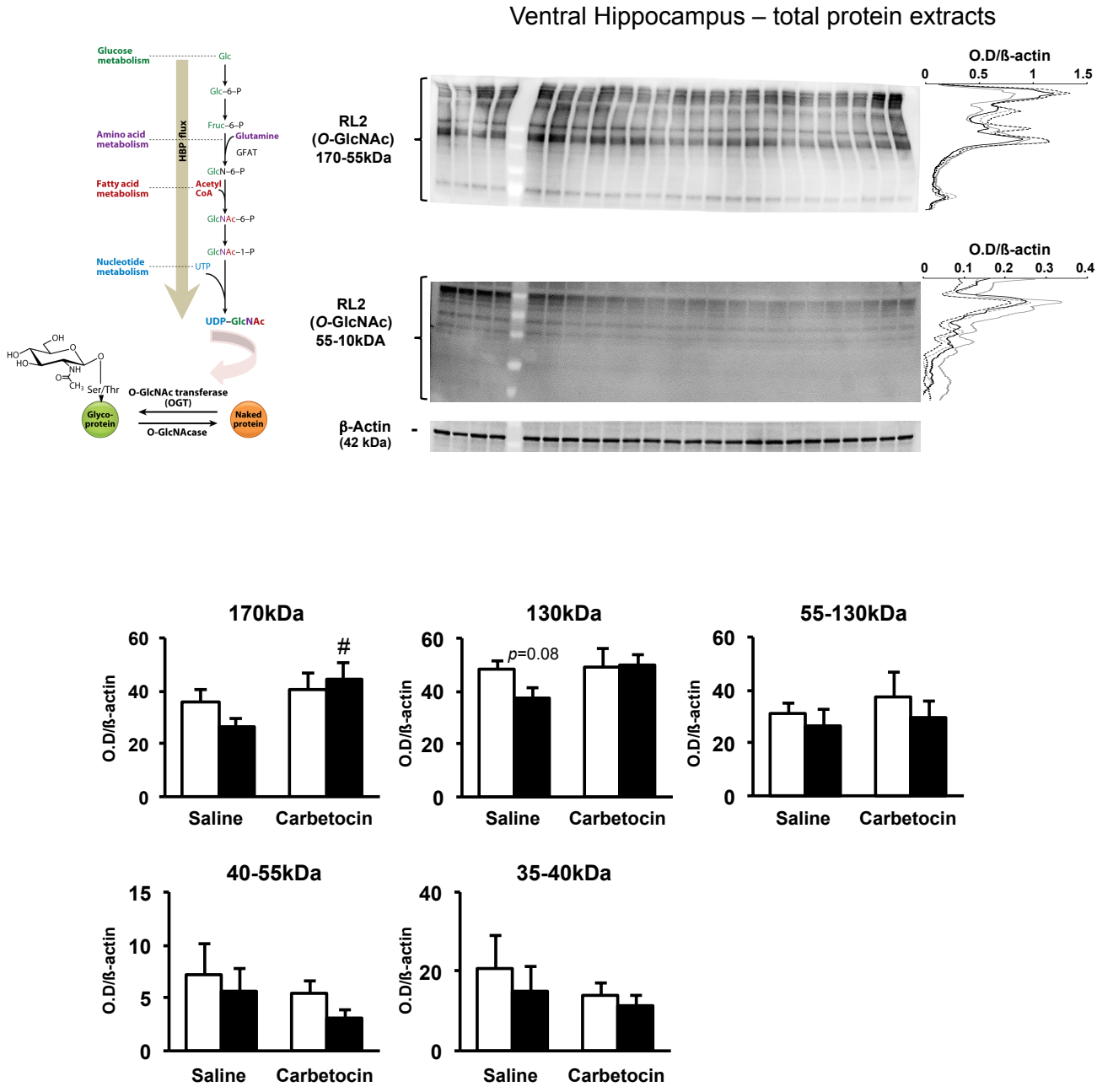

Fig. 9 – Postpartum carbetocin treatment enhances *O*-GlcNAcylation in high-range molecular weight proteins.

Total O-GlcNAc levels were measured in total protein extracts from the ventral hippocampus of the aged offspring of dams treated with carbetocin in the early post-partum period. Values are means ± S.E.M of 6 animals per group. $p < 0.05$ *vs. vs.* the PRS Saline group (#) (Two-Way ANOVA + Fisher's LSD; treatment $F_{1,19} = 4.7$).

2. Evidence for an imbalance between tau *O*-GlcNAcylation and phosphorylation in the hippocampus of a mouse model of Alzheimer's disease

Epidemiological evidence suggests that there is comorbidity between mood disorders and diabetes (Roy and Lloyd, 2012). Recent works also highlight the common cognitive and affective symptoms shared by mood disorders such as depression and AD especially in old age (Bennet and Thomas, 2014; Marine and Boriana, 2014). Indeed, high levels of glucocorticoids play a key role in both pathologies. Clinical studies reported the existence of high levels of glucocorticoids in AD patients (Hartmann et al., 1997; Elgh et al., 2006). Recently, in rat acute model of AD, Brureau and colleagues (2012) showed the existence of a causal relationship between high levels of glucocorticoids and amyloid toxicity. Stress may turn into a factor for further vulnerability to the development of neurodegenerative disorders. Moreover, as discussed above, stress hormones also play a crucial role in the regulation of the energy balance.

Maintenance of glucose homeostasis is essential to ensure adequate nutrient flow to all tissues. As glucose is the main fuel of the CNS, this regulation is particularly important in the brain, which integrates all nutritional information and also actively regulates energy balance (reviewed by Grayson, Seeley and Sandoval, 2013). Metabolism regulation has a high impact on cognitive and mental health (Hendrickx, McEwen, Ouderaa, 2005). Pathophysiological mechanisms resulting from metabolic deregulation may be the common denominator of a range of age-related conditions. Stranaham and Mattson (2012) hypothesized that 'cognitive reserve', i.e. the ability to maintain cognitive performances despite of the development of age-related disorders, rest upon brain metabolic efficiency.

In particular, because of the impairment in insulin signaling (de la Monte, 2012) and brain glucose metabolism AD (Caselli et al., 2008), AD is now likened to "type-3 diabetes" (de la Monte, 2014). Atrophic brain and neurofibrillary deposits are the main features of AD, as first described in 1906 by Alois Alzheimer, a German psychiatrist and neuropathologist (Vishal et al., 2011). AD is indeed characterized by molecular alterations resulting in proteins accumulation and consequent neuronal and synapses loss. Histological observation of AD patient's brain shows the presence of senile plaques and neurofibrillary tangles (Braak and Braak, 1991). Senile plaques are heterogeneous complex mainly composed by beta-amyloid (Aβ) peptides, whereas neurofibrillary tangles are due to tau protein aggregation (Selkoe et al., 2001). According to the amyloid hypothesis, tau alterations occur downstream of Aβ peptides

build up (Hardy and Selkoe, 2002; Selkoe, 2011). These impairments progressively appear in the brain, starting in the hippocampus and then, spreading to the cerebral cortex, causing memory, language and general cognitive deficits (Thal et al., 2002; Hyman, et al., 2012).

2.1. Beta-Amyloid peptides

Amyloid peptides were first isolated in 1984 by Glenner and Wong as a common cerebrovascular protein in Alzheimer and Down's syndrome. Aβ peptides are produced by neuronal cells through the endoproteolytic cleavage of Amyloid Precursor Protein (APP), which normally plays a role in cell–cell and cell–matrix interactions. This amyloidogenic pathway involves specific cleavage enzyme such as β-secretase and γ-secretase (Sisodia and St George-Hyslop, 2002) and mainly leads to the synthesis of Aβ peptides of 40-42 amino acids ($A\beta_{1-42}$) that forms senile plaques. Two different forms of Aβ coexist: monomeric and oligomeric. An equilibrium state between these two structural states is defined, such that below a certain concentration it is primarely monomeric. Moreover, soluble Aβ is mainly monomeric, whereas insoluble oligomers might be synthesized under pathological conditions (Nag et al., 2011). In AD, oligomers have been shown to be toxic by inducing an aberrant cell cycle (Copani et al., 2008) and leading to synaptic dysfunction and neuronal damage (Fig. 22). Conversely, Aβ monomers are neuroprotective and support neuronal survival under condition of trophic deprivation (Giuffrida et al., 2009).

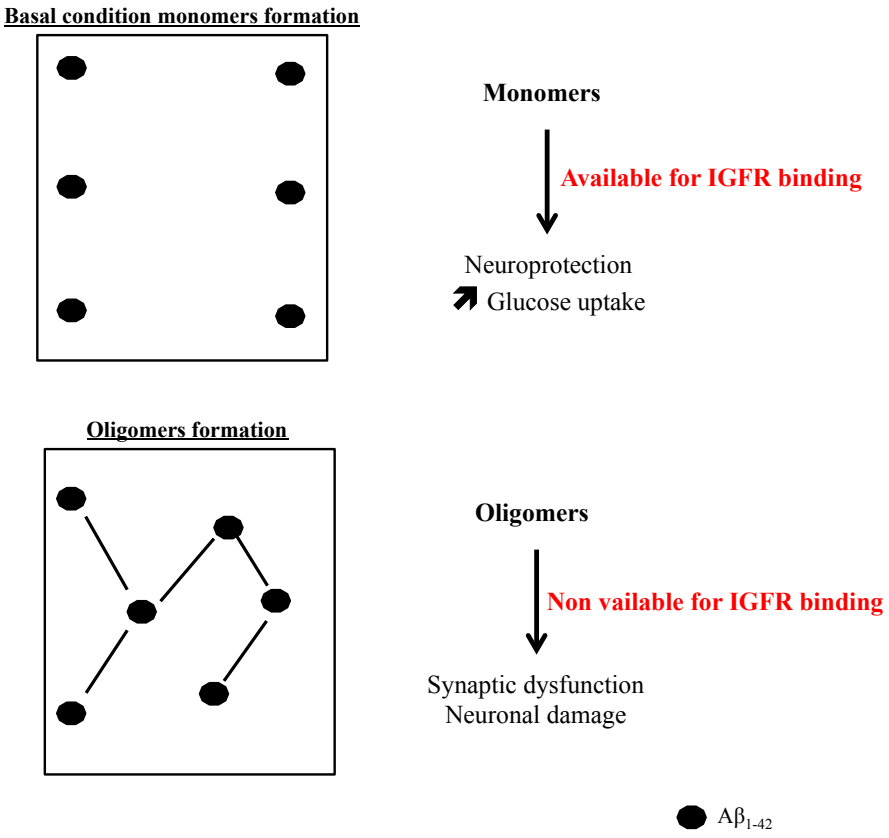

Figure 22: Schematic representation of β-amyloid ($A\beta_{1-42}$) formation in Alzheimer's disease.
The neuronal disorder is characterized by decreased monomers consequent to oligomers formation. Monomers are thus not available for insulin-like growth factor (IGFR) binding finally leading to neuronal damage.

The neuroprotective action of $A\beta_{1-42}$ monomers is mediated by the activation of the phosphatidylinositol-3-kinase (PI3K) pathway, and involved the stimulation of insulin-like growth factor-1 (IGF-1) receptors and/or other receptors of the insulin superfamily. The activation of this pathway leads to Glycogen Synthase Kinase 3β (GSK3β) inhibition, thereby promoting cell survival (Giuffrida et al., 2010). Recent work from Prof. Copani's group demonstrates that $A\beta_{1-42}$ monomers, binding IGF-1 receptors as allosteric modulators, promote Glut3 transporter translocation to the plasma membrane, thus facilitating glucose uptake (Giuffrida et al., 2015; Fig. 23).

2.2. Tau proteins

Tau proteins belong to microtubule-associated proteins. The interaction of tau protein with microtubules is dependent on phosphorylation levels. Indeed, the longest brain tau isoform presents 79 Serines or Threonines phosphorylation putative sites. The different states of Tau phosphorylation are the result of many kinases, part of the proline-directed protein kinases,

which include mitogen activated protein (Drewes et al., 1992), and cyclin-dependent kinases as cdk5 (Baumann et al., 1993); but also non-proline directed kinases such as GSK3β (Hanger et al., 1992), and dual specificity tyrosine phosphorylation regulated kinase (DYRK1a) (Woods et al., 2001). These kinases are abnormally active in Alzheimer's brain and contribute to paired helical filaments formation and neurofibrillary tangles. Thereby, some sites such as Serine 396 or Threonine 205 are specific AD clinical hallmarks.

In the CNS, tau is one of the proteins that undergo *O*-GlcNAcylation at specific Serines (Ser) and Threonines (Thr) residues that can be also phosphorylated, including Ser396 and Thr205 (Wang, Grundke-Iqbal and Iqbal, 1996; Takahashi et al., 1999; Lefebvre et al., 2003b). tau glycosylation and phosphorylation are two mutually exclusive post-translational modifications (Arnold et al., 1996), suggesting that an impaired *O*-GlcNAcylation might cause tau hyperphosphorylation (Lefebvre et al., 2003a,b; Yuzwa et al., 2014; Fig. 22). Also, pharmacological inhibition of OGT and OGA (i.e., the enzymes catalyzing the addition or removal of *O*-GlcNAc residues on targeted protein) could regulate tau aggregation (Yuzwa et al., 2014; Lim et al., 2015). These data suggest a crucial role for *O*-GlcNAcylation in the protective mechanisms for protein aggregation, which is disrupted in neurodegenerative diseases (Marotta et al., 2015).

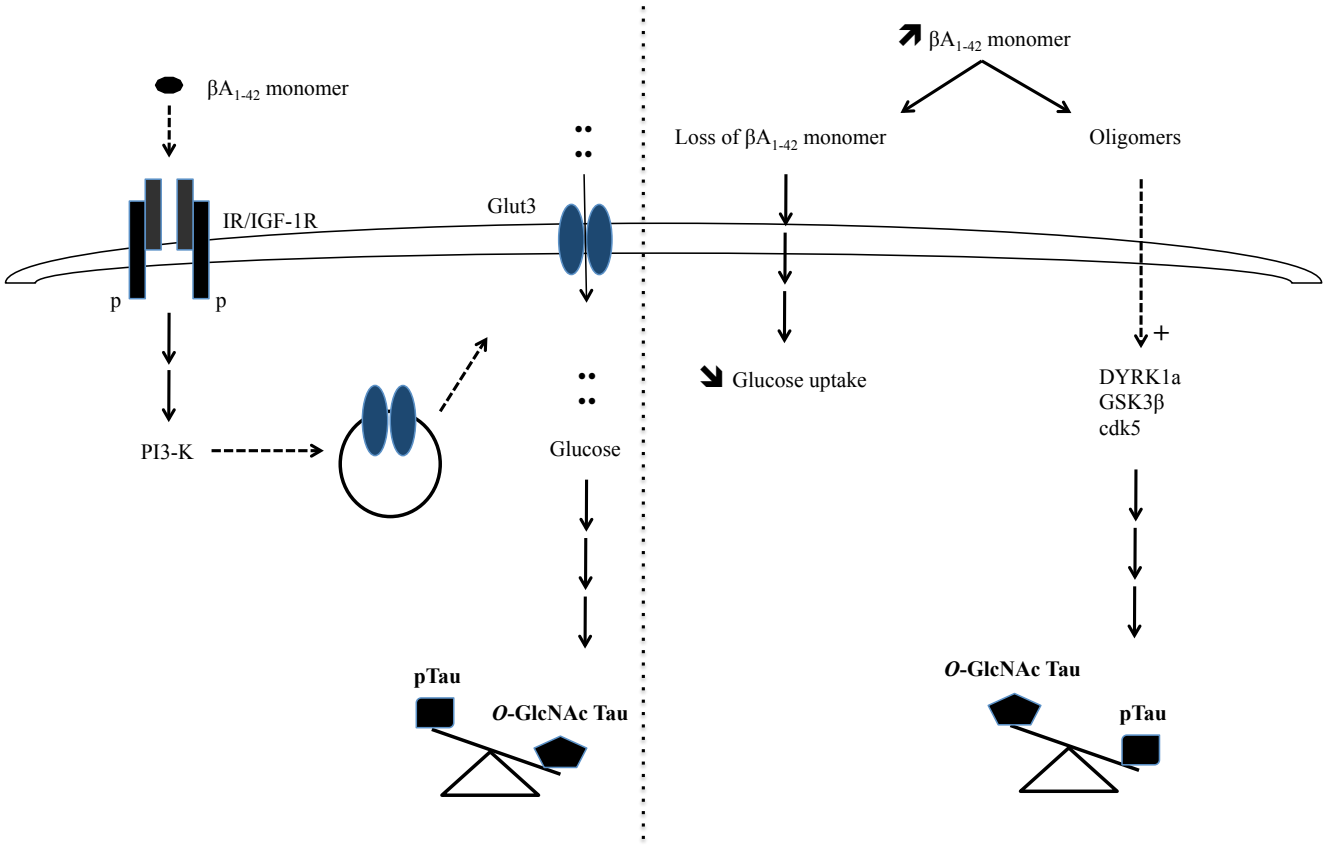

Figure 23: Possible interactions between β-Amyloid monomers ($A\beta_{1-42}$) and the insulin/IGF1 receptor (IR/IGF-1R).

$A\beta_{1-42}$ monomers released at the synapse (left panel) promote the activation of the insulin/IGF-1 signaling pathway resulting into: *i*) sustained neuron survival (through PI3K pathway activation) and neuronal glucose provision via Glut3 translocation *ii*) decreased tau phosphorylation (via Glycogen Synthase Kinase 3β (GSK3β) inhibition). On the other hand, accumulating Aβ oligomers (right panel) leads to increased Tau protein phosphorylation through activation of kinases: dual specificity tyrosine phosphorylation regulated kinase (DYRK1a), GSK3β, cyclin-dependent kinases (cdk5). Dashed lines refer to not proven mechanism (adapted from Giuffrida et al., 2015).

The aim of this work was to investigate whether a perturbed balance between tau *O*-GlcNAcylation and phosphorylation might contribute to the pathophysiology of AD. To our knowledge, there is no evidence that tau is hypo-*O*-GlcNAcylated in the brain of AD transgenic mice. Thus, we decided to use 3XTg-AD mice, which are a murine model of AD carrying mutations of presenilin-1 ($PS1_{M146V}$), amyloid precursor protein (APP_{Swe}), and tau_{P301L}. 3xTg-AD mice develop extracellular Aβ deposits prior to tangle formation consistent with the amyloid cascade hypothesis. This model presents elevated face and construct validity because 3XTg-AD mice develop an age-dependent and progressive neuropathology, presenting both hyperphosphorylated tau protein (Oddo et al., 2003) and cognitive decline (Singh et al., 2012;

Knight et al., 2014; Yeung et al., 2015), combined with an age-related reduction in glucose uptake (Nicholson et al., 2010). In addition, 3xTg-AD mice display a reduced level of blood glucose after fasting (Rothman et al., 2012).

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Evidence for an imbalance between tau *O*-GlcNAcylation and phosphorylation in the hippocampus of a mouse model of Alzheimer's disease

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Abstract

Intracellular accumulation of hyperphosphorylated tau protein is linked to neuronal degeneration in Alzheimer's disease (AD). Mounting evidence suggests that tau phosphorylation and *O*-N-acetylglucosamine glycosylation (*O*-GlcNAcylation) are mutually exclusive post-translational modifications. *O*-GlcNAcylation depends on 3-5% of intracellular glucose that enters the hexosamine biosynthetic pathway. To our knowledge, the existence of an imbalance between tau phosphorylation and *O*-GlcNAcylation has not been reported in animal models of AD, as yet. Here, we used triple transgenic (3xTg-AD) mice at 12 months, an age at which hyperphosphorylated tau is already detected and associated with cognitive decline. In these mice, we showed that tau was hyperphosphorylated on both Ser396 and Thr205 in the hippocampus, and to a lower extent and exclusively on Thr205 in the frontal cortex. Tau *O*-GlcNAcylation, assessed in tau immunoprecipitates, was substantially reduced in the hippocampus of 3xTg-AD mice, with no changes in the frontal cortex or in the cerebellum. No changes in the expression of the three major enzymes involved in *O*-GlcNAcylation, i.e., glutamine fructose-6-phosphate amidotransferase, *O*-linked β-N-acetylglucosamine transferase, and *O*-GlcNAc hydrolase were found in the hippocampus of 3xTg-AD mice. These data demonstrate that an imbalance between tau phosphorylation and *O*-GlcNAcylation exists in AD mice, and strengthens the hypothesis that *O*-GlcNAcylation might be targeted by disease modifying drugs in AD.

Keywords: *O*-GlcNAcylation; 3xTg-AD mice; tau protein; hippocampus

1. Introduction

An impairment of brain glucose uptake is invariably associated with Alzheimer's disease (AD) and precedes by several decades the clinical onset of AD (Small et al., 1995; Reiman et al., 1996; Cunnane et al., 2011; Ossenkoppele et al., 2012). Because the defect in glucose uptake likely reflects a condition of central insulin/insulin-like growth factor resistance (Steen et al., 2005; Giuffrida et al., 2010), AD has been considered as a localized form of diabetes ("type-3 diabetes") (reviewed by De La Monte and Tong, 2014). The defect in glucose consumption can make neurons vulnerable to damage *via* a number of mechanisms that include a general impairment of energetic metabolism and a defective hexosamine biosynthetic pathway (HBP)-dependent glycosylation. Three to five per cent of the glucose that enters the cell is utilized for HBP-dependent glycosylation. Glucose is converted into UDP-N-acetylglucosamine (UDP-GlcNAc), which acts as a GlcNAc donor in *O*-N-acetylglucosaminylation (*O*-GlcNAcylation) reactions. The enzyme *O*-linked β-N-acetylglucosamine transferase (OGT) transfers GlcNAc residues to protein substrates in all cellular compartments, whereas *O*-GlcNAc hydrolase (OGA) catalyzes the removal of the sugar (reviewed by Hart et al., 2011).

Similarly to other cytoplasmic proteins (Hart et al., 2011; Hanover et al., 2012), tau undergoes *O*-GlcNAcylation at specific serine (Ser) and threonine (Thr) residues that can also be phosphorylated, including Ser396 and Thr205 (Wang et al., 1996; Takahashi et al., 1999; Lefebvre et al., 2003). Tau *O*-GlcNAcylation and phosphorylation are mutually exclusive, suggesting that an impaired *O*-GlcNAcylation might cause tau hyperphosphorylation and aggregation (Arnold et al., 1996; Liu et al., 2002; Lefebvre et al., 2003; 2005; Kang et al., 2013; Yuzwa et al., 2014a; Lim et al., 2015). If so, a reduction in tau *O*-GlcNAcylation might contribute to the pathophysiology of AD because hyperphosphorylated tau gives rise to neurofibrillary tangles, which are found in the AD brain and lie at the core of neurodegenerative processes associated with AD (reviewed by Selkoe, 2001). A recent study also suggests that changes in *O*-GlcNAcylation levels may also influence β-amyloid pathology, which is a second hallmark of the AD brain, in the presence of tau pathology (Yuzwa et al., 2014b). Besides its putative relevance in the pathophysiology of AD, recent findings demonstrate the crucial involvement of *O*-GlcNAcylation in the modulation of synapses activity (Tallent et al., 2009; Din et al., 2010; Taylor et al., 2014), especially in the hippocampus, a crucial structure for memory processes. This lays the groundwork for a link between hippocampus metabolic status, synaptic plasticity and cognitive performances through multiple proteins *O*-GlcNAcylation.

To our knowledge, the balance between tau *O*-GlcNAcylation and phosphorylation has never been studied in the brain of mice modeling AD. Here, we used the model of triple transgenic mice (3xTg-AD) developed by LaFerla and colleagues. This model harbors three mutant genes: presenilin-1 ($PS1_{M146V}$), Amyloid Precursor Protein (APP_{Swe}), and tau_{P301L} transgenes (Oddo et al., 2003), and recapitulates all major hallmarks of AD, with an age-dependent central increase of both amyloid deposition and intracellular neurofibrillary tangles (Oddo et al., 2003, 2007) associated with progressive cognitive decline. Cognitive decline is characterized by a progressive impairment of hippocampus-related spatial memory, which begins at 6-8 months of age (Billings et al., 2005; Nelson et al., 2007) and is fully established at 12-14 months of age (Carroll et al., 2007; Clinton et al., 2007; Gimenez-Llort et al., 2007; Arsenault et al., 2011; Filali et al., 2012;). 3xTg-AD mice also show anxiety- and depressive-like behaviors, which are less age-dependent (Billings et al., 2005; Clinton et al., 2007; Nelson et al., 2007; Filali et al., 2012; Romano et al., 2014). A particular feature that makes 3xTg-AD mice a valuable model for the study of *O*-GlcNAcylation is that these mice develop an age-related reduction in brain glucose uptake (Nicholson et al., 2010). We now report that 12 month-old 3xTg-AD mice show a reduced tau *O*-GlcNAcylation associated with tau hyperphosphorylation in the hippocampus. Reduction of tau *O*-GlcNAcylation was region-specific and was not associated with changes in the expression and activity of enzymes that regulate protein *O*-GlcNAcylation.

2. Materials & Methods

2.1.Animals

We used 12 month-old 3xTg-AD male mice harboring presenilin 1 ($PS1_{M146V}$), amyloid-precursor protein (APP_{Swe}), and tau_{P301L} mutations (Oddo et al., 2003). Age- and gender-matched *wild-type* (WT) animals were used as control. Animals were bread at the vivarium of the Puglia and Basilicata Experimental Zooprophylactic Institute (Foggia, Italy). Genotypes were confirmed by polymerase chain reaction (PCR) after tail biopsies (Oddo et al., 2003). Animals were housed at 22°C with a 12 h light/dark cycle and food and water *ad libitum*.

2.2.Western Blot Analysis

Mice were killed by cervical dislocation. The hippocampus, frontal cortex and cerebellum were rapidly dissected and immediately stored at -80°C. Tissues were homogenized at 4°C in a lysis buffer containing 320 mM sucrose, 5 mM HEPES, pH 7.4, 500 mM NaF, 10% SDS, 80 mM streptozotocine, and phosphatase and protease inhibitors. The bicinchoninic acid (BCA) assay

was used for the determination of protein concentrations. Homogenized tissues were resuspended in Laemmli reducing buffer, and 20 µg of proteins were separated by electrophoresis on Criterion TGX 4-15% precast SDS-polyacrylamide gels (BioRad), and 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-p-tauSer396 (1:5000; Santa Cruz Biotechnology, #sc-101815), rabbit polyclonal anti-p-tauThr205 (1:1000; Santa Cruz Biotechnology, #sc-101817), rabbit polyclonal anti-pan-tau (1:2000; Santa Cruz Biotechnology catalog #sc-5587), rabbit polyclonal anti-GFAT (1:300; Santa Cruz #sc-134894); mouse monoclonal anti-*O*-GlcNAc (1:2000, RL2; ThermoFisher Scientific; #MA1-072), rabbit polyclonal anti-OGT AL35 (1:2000; generously provided by G. W. Hart, Johns Hopkins University, Baltimore, MD, USA), chicken anti-OGA 345 (1:2000; generously provided by G. W. Hart) and mouse monoclonal anti-β-actin (dilution 1:80.000, Sigma-Aldrich). All primary antibodies were incubated overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary anti-mouse or anti-rabbit antibodies (purchased from GE Healthcare) were used at a dilution from 1:10000 to 1:20000 and were incubated for 1 h at room temperature. Enhancing chemiluminescence detection system (Pierce ECL,Thermo Scientific) was used for detection on autoradiography films (GE Healthcare). Signals obtained for tau, p-tau and β-Actin levels were quantified with GS-800 scanner (BioRad) associated to Quantity One software (BioRad).

Densitometric lane spetra profiles obtained for total *O*-GlcNAcylated proteins were generated by measuring the optical density (O.D.) of each line of pixel of a lane using a custom-built macro in ImageJ software.

2.3.Immunoprecipitation

Tissue was lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) containing phosphatase inhibitors, protease inhibitors and streptozotocin (80 mM). Protein concentrations were determined by the BCA assay. In order to avoid non specific binding, protein A sepharose beads (GE Healthcare) were first washed with RIPA buffer. After centrifugation, supernatant was discarded and protein A sepharose beads were incubated with protein extracts (0.5 mg per sample) for 2 h at 4°C for pre-clearing. After centrifugation (4000 g), extracts were incubated overnight with the pan-tau antibody (2 µg). Antibody-antigen complexes were incubated for 2 h with 30 µL of protein A beads before centrifugation (10 000g). Samples were then resuspended in Laemmli reducing

buffer before SDS-PAGE (see Western Blot Analysis section). The secondary antibody used for the detection of pan-tau and p-tau was an anti-rabbit True Blot® immunoglobulin (eBioscience), which limits the interference with the immunoprecipitating IgG heavy and light chains. The secondary antibody used for the detection of *O*-GlcNAc was a HRP-conjugated anti-mouse antibody (dilution 1:10.000, GE Healthcare).

2.4.OGT and OGA Activity Assays

Hippocampi of 3xTg-AD and wt mice were homogenized in buffer consisting of 50 mM Tris-Hcl (pH 7.4), 2 mM EDTA, 10 mM β-mercaptoethanol, 8,5% sucrose, phosphatase and protease inhibitors (Sigma Aldrich®). Resulting homogenates were first centrifuged at 16 000 g at 4°C for 10 minutes. 2 volumes of 30% polyethylene glycol 8000 (in 25 mM HEPES containing 10 mM $MgCl_2$, pH 7,23) was added to supernatants. Samples were subsenquently centrifuged at at 16 000 g at 4°C for 10 minutes. The pellets obtained were resuspended in OGT assay buffer (25mM HEPES, pH 7.5, 10 mM $MgCl_2$ and 1mM EDTA). Extracts were then incubated 2h at 24°C with the addition of 0.4 µCi of UDP-[^{3}H]GlcNAc in the OGT assay buffer. After labeling, proteins were precipitated with trichloro-acetic acid (TCA) at a final concentration of 20%, and then recovered on glass microfiber filters (GF/A, Whatman), and intensively washed with 0.1% TCA under vacuum. The precipitates were rinsed with absolute ethanol and then counted on the filters in a liquid scintillation counter (Beckman). Each experiment was performed in triplicate.

OGA activities were determined in hippocampi homogenates using the method described in Liu et al., 2012. Briefly, hippocampi homogenates (100µl/assay) were incubated 30 min at 37°C in a buffer containing 50 mM sodium cacodylate (pH6.4), 2 mM p-nitro-phenyl-N-acetyl-β-D-glucosaminide, 0.3% bovine serum albumin, 2 mM β-mercaptoethanol, 2mM EDTA, phosphatase and protease inhibitors (Sigma Aldrich®). Reactions were stopped with the addition of 0.9 ml of 0.5 M sodium carbonate, and absorbance was measured at 400nm. Each experiment was performed in triplicate.

2.5.Statistics

Statistical differences between groups were determined with Student's t test. Correlations were performed using the Pearson's correlation analysis. Significance was set to $p < 0.05$.

3. Results

3.1.Enhanced tau phosphorylation in 3xTg-AD mice

We measured the levels of tau protein and its phosphorylated forms at Ser396 or Thr205, in the hippocampus, frontal cortex and cerebellum of 3xTg-AD mice and their WT counterparts.

In the hippocampus, 3xTg-AD mice showed >2 fold increase in total tau (pan-tau) protein levels (t_{32}=9.02; p<0.001; Fig.1A), and also significant increases in p-tauSer396 (t_{32}=2.14; p<0.05; Fig. 1B) and p-tauThr205 (t_{32}=7.68; p<0.001; Fig. 1C), if levels were normalized by β-actin (Fig. 1A-C). Phosphorylated tau levels in 3xTg-AD mice were significantly reduced if normalized by the amount of pan-tau (p-tauSer396/pan-tau: t_{32}=-6.73; p<0.001; p-tauThr205/pan-tau: t_{32}=-3.11; p<0.05; Fig. 1D, E) because of the substantial accumulation of tau protein in the hippocampus of these mice. We believe that normalization of phosphorylated tau with respect to β-actin levels is more reliable for two reasons: (*i*) pan-tau levels incorporate different forms of phosphorylated tau that may be increased in 3xTg-AD mice; and (*ii*) phosphorylation may reduce the turnover rate of tau, thus increasing tau levels in brain tissue.

In the frontal cortex and cerebellum, 3xTg-AD mice also show significant increases in pan-tau protein levels (t_8=3.661; p<0.01 and t_8=4.516; p<0.01, respectively). However, this increase was much smaller then that found in the hippocampus (43% and 44% increase in the frontal cortex and cerebellum, respectively; Fig 2A and F). In the frontal cortex of 3xTg-AD mice p-tauThr205 levels were significantly increased if normalized by β-actin (t_8=3.152; p<0.05; Fig. 2C), but not if normalized by pan-tau (Fig. 2E). No significant changes were found in p-tauSer396 (Fig. 2B, D). In the cerebellum of 3xTg-AD mice, no significant changes in p-tau levels were found, although a trend to an increase in p-tauSer396 and p-tauThr205 was seen if levels were normalized by β-actin (t_8=2.283; p=0.052 and t_8=2.266; p=0.053, respectively) (Fig. 2G, H).

3.2.Overall protein *O*-GlcNAcylation is decreased in the hippocampus and cerebellum of 3xTg-AD mice

Using an anti-*O*-GlcNAc antibody, we first measured levels of all *O*-GlcNAcylated proteins with molecular size over 55 kDa. The optical density (O.D.) of all detectable bands of the immunoblots was measured. 3xTg-AD mice showed a significant reduction in the overall protein *O*-GlcNAcylation in the hippocampus (t_8=6.367; p<0.001) and cerebellum (t_8=3.634; p<0.01), but not in the frontal cortex (t_8=-0.051; p=0.960) (Fig. 2A-C). In the molecular weight range corresponding to the size of tau protein (60-70kDa), a reduced *O*-GlcNAcylation was

exclusively found in the hippocampus of 3xTg-AD mice (t_8=2.678; p<0.05; see Fig. 3A). Interestingly, there was no change in the expression of GFAT, OGT, and OGA in any brain region (Fig. 4A-C), indicating that the reduction of *O*-GlcNAcylation found in the hippocampus and cerebellum of 3xTg-AD mice was not due to an altered expression of the rate-limiting enzyme of the HBP, nor of the enzyme regulating *O*-GlcNAcylation.

3.3.Reduced tau *O*-GlcNAcylation in the hippocampus of 3xTg-AD mice

To specifically examine whether *O*-GlcNAcylation of tau protein was altered in 3xTg-AD mice, we performed immunoprecipitation experiments with an anti-tau polyclonal antibody. We adopted this strategy because no anti-*O*-GlcNAc-tau antibodies are currently available.

We performed two experiments using different mice. Mice of experiment 1 were used for measurements of phosphorylated and *O*-GlcNAcylated tau in tau immunoprecipitates from the hippocampus, frontal cortex, and cerebellum. Mice of experiment 2 were used for measurements of *O*-GlcNAcylated tau exclusively in hippocampal tau immunoprecipitates.

In hippocampal immunoprecipitates from 3xTg-AD mice of experiment 1, pan-tau, p-tauSer396 and p-tauThr205 levels were significantly increased (t_8=3.410; p<0.01; t_8=3.188; p<0.05 and t_8=4.428; p<0.01, respectively; Fig. 5A-C), whereas *O*-GlcNAcylated tau levels were reduced by >45% (t_8=-3.139; p<0.05; Fig. 5 D). When *O*-GlcNAcylated tau levels of experiment 1 data were normalized by p-tauSer396 or p-tauThr205 levels, the reduction of tau *O*-GlcNAcylation was even more prominent (>65%; t_8=-2.797; p<0.05 and t_8=-4.56; p<0.01, respectively) (Fig. 5E, F). There was a highly significant inverse correlation between p-tauSer396 and *O*-GlcNAcylated tau levels in the hippocampus (p-tauSer396 x *O*-GlcNAc, r=-0.8819; p<0.001), whereas no correlation was found between p-tauThr205 and *O*-GlcNAcylated tau (Fig. 5G).

Data of exepriments 1 and 2 were combined for the evaluation of tau *O*-GlcNAcylation with a stronger statistical power. Using this approach, the reduction of tau *O*-GlcNAcylation in 3xTgAD mice was fully confirmed with an extent even greater than that observed with data of experiment 1 alone (pan-tau: t_{20}=3.125; p<0.01; Fig. 5H; *O*-GlcNAcylated tau: t_{20}=-5.144; p<0.001; Fig. 5I).

No significant changes in phosphorylated or *O*-GlcNAcylated tau were found in immunoprecipitates from the frontal cortex or the cerebellum of 3xTg-AD mice (Fig. 6). However, we found a direct correlation between p-tauThr205 and *O*-GlcNAcylated tau levels in frontal cortex (p-tauThr205 x *O*-GlcNAc, r=0.8103; p<0.05; Fig. 6G) and an inverse

correlation between p-tauSer396 and *O*-GlcNAcylated tau levels in the cerebellum (p-tauSer396 x *O*-GlcNAcylated tau, r=-0.6547; p<0.05).

3.4 OGT and OGA activities are not modified in the hippocampus of 3xTg-AD mice

The activity of enzymes regulating *O*-GlcNAcylation was assessed in hippocampus of 3xTg-AD and WT mice. OGT activity was unchanged in 3xTg-Ad mice (t_7=-0.418; p=0.689; Fig. 7A). OGA activity was not significantly enhanced in 3xTg-Ad mice mice (t_{10}=1.594; p=0.142; Fig. 7B).

4. Discussion

We used mice harboring human mutations of APP, PS1, and tau (3xTg-AD mice), which are considered as a putative mouse transgenic model of AD. We found a selective reduction of tau *O*-GlcNAcylation in the hippocampus of these mice, which was associated with the expected tau hyperphosphorylation (Oddo et al., 2003, 2007). To our knowledge, this is the first evidence that tau is hypo-*O*-GlcNAcylated in a mouse model of AD.

Although increasing evidence suggests that abnormalities of *O*-GlcNAcylation play a role in the pathophysiology of AD, studies on *O*-GlcNAcylation in the AD brain have produced contrasting results. Liu et al. (2009) have found a reduced tau *O*-GlcNAcylation in brain tissue from AD patients,–whereas increases in protein *O*-GlcNAcylation were found in two other reports (Griffith et al., 1995; Förster et al., 2014). There might be many explanations for the contrasting data obtained in the AD brain, including differences in the selected brain regions, sample preparation, type of anti-*O*-GlcNAc antibody, and, more importantly, the post-mortem interval before tissue sampling. It has been demonstrated that *O*-GlcNAcylation levels decline with post-mortem delay of brain tissue (Liu et al., 2004). Here, the reduction of tau *O*-GlcNAcylation levels was only found in the hippocampus of 3xTg-AD mice, which harbor a tau mutation (tau_{P301L}), in addition to the APP and PS1 mutations. Steady-state levels of transgene-derived human tau protein are high in the hippocampus and cerebral cortex, and low in the cerebellum (Oddo et al., 2003). Hence, the evidence that the imbalance between tau *O*-GlcNAcylation and phosphorylation was observed in the hippocampus, but not in the cortex, indicates that expression of transgenic tau did not affect our results.

In 3xTg-AD mice, tau pathology appears first in the hippocampus, where it is already visible at 12 months of age, and later spreads to the cortex (Oddo et al., 2003; Mastrangelo and Bowers, 2008). Thus, the selective reduction in tau *O*-GlcNAcylation found in the hippocampus of 12-

month-old 3xTg-AD mice is in line with the pattern of tau pathology in these mice. Several mechanisms may account for the reduction of tau *O*-GlcNAcylation in the hippocampus of 3xTg-AD mice, such as a primary phosphorylation of tau, a defective expression/activity of HBP enzymes, and a reduction in glucose uptake in neurons. These three hypotheses are not mutually exclusive. An increased phosphorylation might be directly linked to the reduction in tau *O*-GlcNAcylation because, in tau immunoprecipitates, glycosylation was reduced only in the region in which phosphorylation was enhanced, i.e. in the hippocampus. However, the general defect of protein *O*-GlcNAcylation found in the hippocampus suggests that a reduced *O*-GlcNAcylation might be a primary event, facilitating tau phosphorylation on hypoglycosylated Ser or Thr residues. Expression of the HBP rate-limiting enzyme, GFAT, as well as the expression of enzymes regulating *O*-GlcNAcylation, i.e. OGT, and OGA, did not change in the hippocampus of 3xTg-AD mice, suggesting that the pathway was not constitutively defective in these mice. Remarkably, the activity of OGT and OGA was not modified in these animals. Hence, a primary defect in glucose uptake might be responsible for the reduced *O*-GlcNAcylation. Accordingly, Ding et al. (2013) have found a reduction in brain glucose uptake associated with a decreased expression of the type-3 neuronal glucose transporter (GLUT-3) in 12-month-old 3xTg-AD mice. We were intrigued from the finding that tau *O*-GlcNAcylation and phosphorylation was unchanged in cerebellar immunoprecipitates in spite of the reduction in protein *O*-GlcNAcylation observed in cerebellar homogenates of 3xTg-AD mice. This suggests that an overall reduction in *O*-GlcNAcylation might contribute to, but is not sufficient for, tau hyperphosphorylation in 3xTg-AD mice. It is likely that the balance between tau *O*-GlcNAcylation and phosphorylation is regulated by multiple mechanisms that include the intracellular availability of UDP-GlcNAc and the region-specific activation of protein kinases that phosphorylate tau, such as glycogensynthase kinase-3β (GSK-3β) and type-5 cyclin-dependent kinase (Hanger et al., 1992; Baumann et al., 1993). Perhaps it is only in the hippocampus that these different mechanisms converge in 12-month-old 3xTg-AD mice.

The reduction of *O*-GlcNAcylation we have found in the hippocampus of 12-month-old 3xTg-AD mice may have a deep impact on the pathological phenotype of these mice contributing to tau aggregation, synaptic dysfunction, behavioral abnormalities, and neurodegeneration (Webster et al., 2014). Of note, *O*-GlcNAcylation may modulate mechanisms of activity-dependent synaptic plasticity, e.g., long-term potentiation (LTP) and long-term-depression (LTD) in the hippocampus by regulating the expression and activity of AMPA receptors and synapsin I (Tallent et al., 2009; Din et al., 2010; Skorobogatko et al., 2014; Taylor et al., 2014).

Whether, and to what extent, the reduced protein *O*-GlcNAcylation has any role on the impairment of hippocampal synaptic plasticity and spatial memory observed in old 3xTg-AD mice (Oddo et al., 2003; Billings et al., 2005; Clinton et al., 2007; Carroll et al., 2007; Gimenez-Llort et al., 2007; Nelson et al., 2007; Arsenault et al., 2011; Filali et al., 2012) is an interesting question that warrants further investigation. Although a correlation between tau hyperphosphorylation and cognitive decline in 3xTg-AD mice has not been demonstrated so far (Caccamo et al., 2007; Oddo et al., 2007; Parachikova et al., 2010; Baglietto-Vargas et al., 2014), a reduced tau *O*-GlcNAcylation leading to tau hyperphosphorylation might contribute to the impairment of activity-dependent synaptic plasticity associated with AD. Accordingly, tau phosphorylation at Serine 396 is required for hippocampal LTD (Regan et al., 2015), and tau hyperphosphorylation at epitopes recognized by PHF-1 (Ser 396/404) and AT8 (Ser199/202-Thr205) is enhanced in the hippocampus following LTD induction (Mondragon-Rodríguez et al., 2012). In addition, activation of tau-phosphorylating enzyme, GSK-3β, (Jo et al., 2011; Shipton et al., 2011) and tau phosphorylation (Shipton et al., 2011) are required for the impairment of hippocampal LTP caused by β-amyloid$_{1-42}$ (Aβ_{1-42}). Thus, a reduced tau *O*-GlcNAcylation might be a primary mechanism in the chain of events favoring synaptic weakening over synaptic potentiation in response to extracellular aggregates of Aβ_{1-42}. It will be interesting to examine whether drugs that enhance *O*-GlcNAcylation, such as the OGA inhibitor, Thiamet G, slow the progression of AD-related pathology and the impairment of synaptic plasticity underlying cognitive dysfunction in 3xTg-AD mice, and whether these drugs improve cognitive dysfunction in 3xTg-AD mice. Interestingly, systemic administration of Thiamet G, has been found to reduce tau pathology, slow neurodegeneration, and prolong survival in mouse models of tauopathies (Yuzwa et al., 2012; Borghgraef et al., 2013; Graham et al., 2014), and to prevent cognitive decline and amyloid deposits in the bigenic tau/APP mutant mice (Yuzwa et al., 2014b).

5. Conclusions

Our data demonstrate that an imbalance between tau phosphorylation and *O*-GlcNAcylation exists specifically in the hippocampus of 3xTg-AD mice. This defect in proteins *O*-GlcNAcylation, including tau, found in the hippocampus might be an upstream causative factor contributing to cognitive decline. This strengthens the hypothesis that *O*-GlcNAcylation might be targeted by disease modifying drugs in AD.

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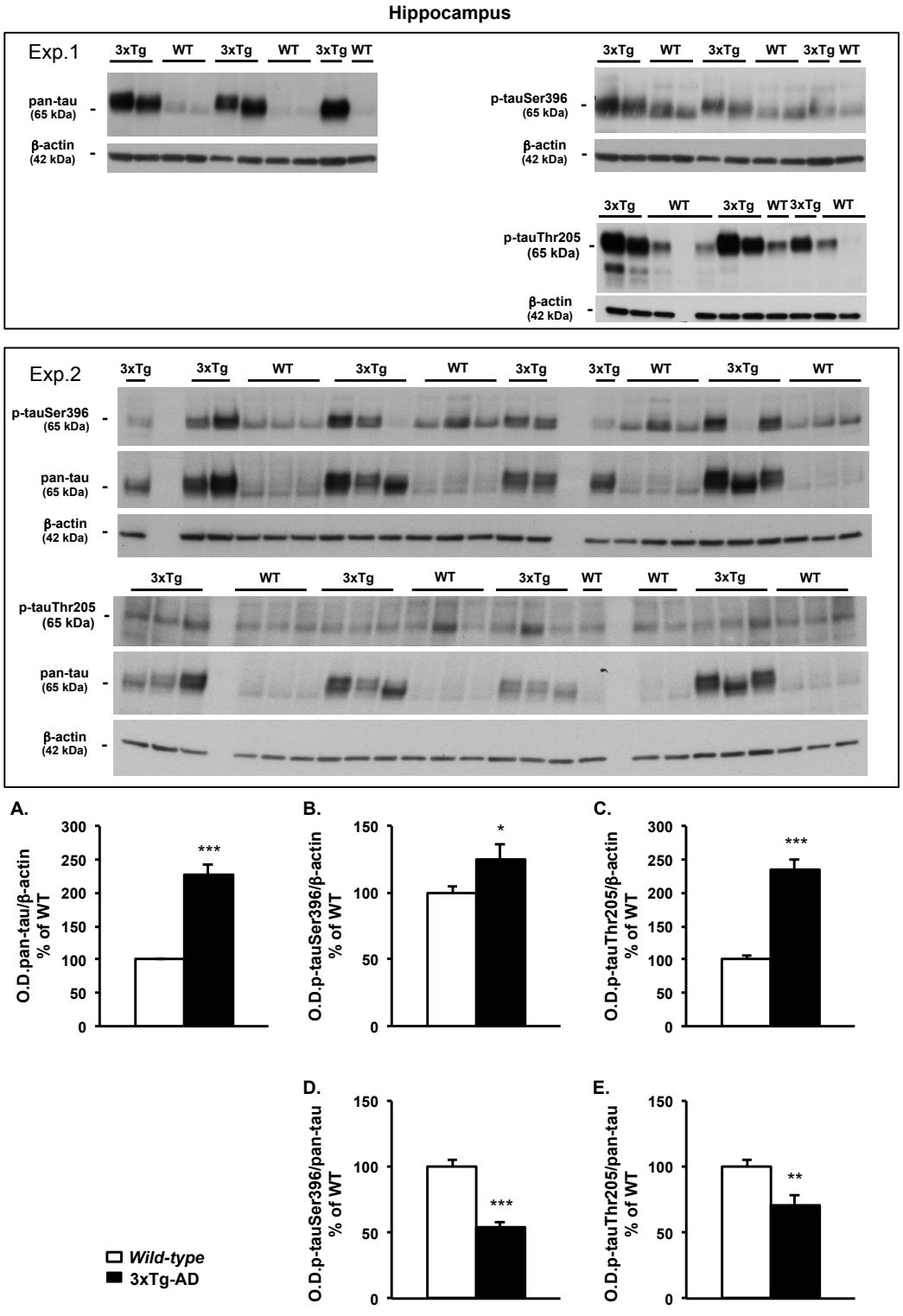

Figure 1 – Enhanced pan-tau and phosphorylated tau levels in the hippocampus of 3xTg-AD mice. Pan-tau, p-tauSer396 and p-tauThr205 levels were measured by immunoblot in experiment 1 (n=5 per group) and 2 (n=12 per group), and values (means ± S.E.M.) were expressed as percent of the respective groups of *wild-type* mice. Values were normalized by ß-actin (A, B and C) or pan-tau (D and E). $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***) vs. the respective *wild-type* mice (Student's t test). In experiment 2, pan-tau values were obtained from the same extracts run in two different immunloblots. Each single pan-tau value is the average of the two determinations in the two blots (one under p-tauSer396 and the other under p-tauThr205).

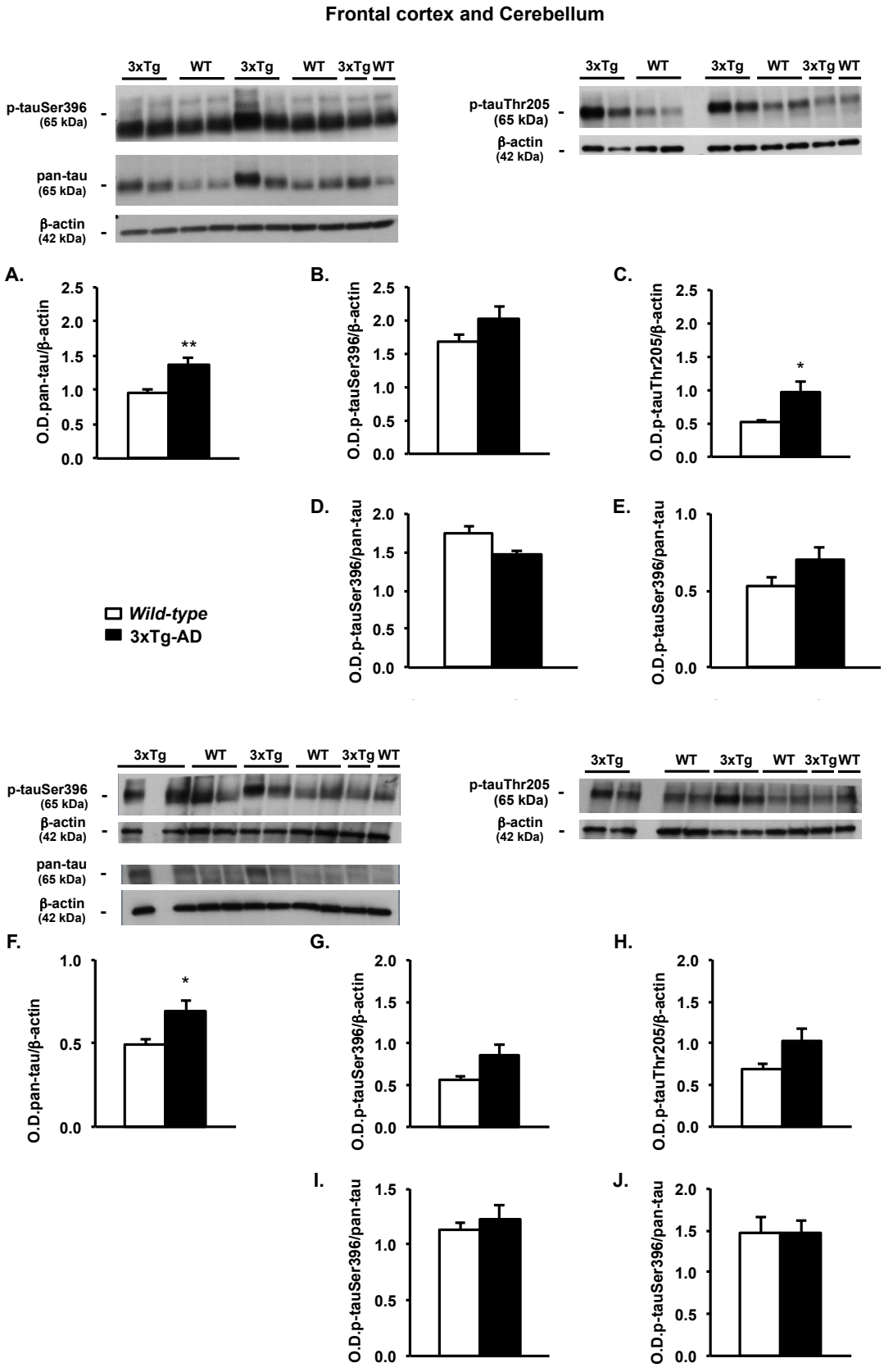

Figure 2 – Tau and phosphorylated-tau levels were in the frontal cortex and cerebellum of 3xTg-AD mice. Values are means ± S.E.M. of 5 determinations per group and were normalized by ß-actin (A, B, C, F, G, H) or pan-tau (D,E, I, J). $p<0.05$ (*), $p<0.01$ (**) vs. the respective *wild-type* mice (Student's t test).

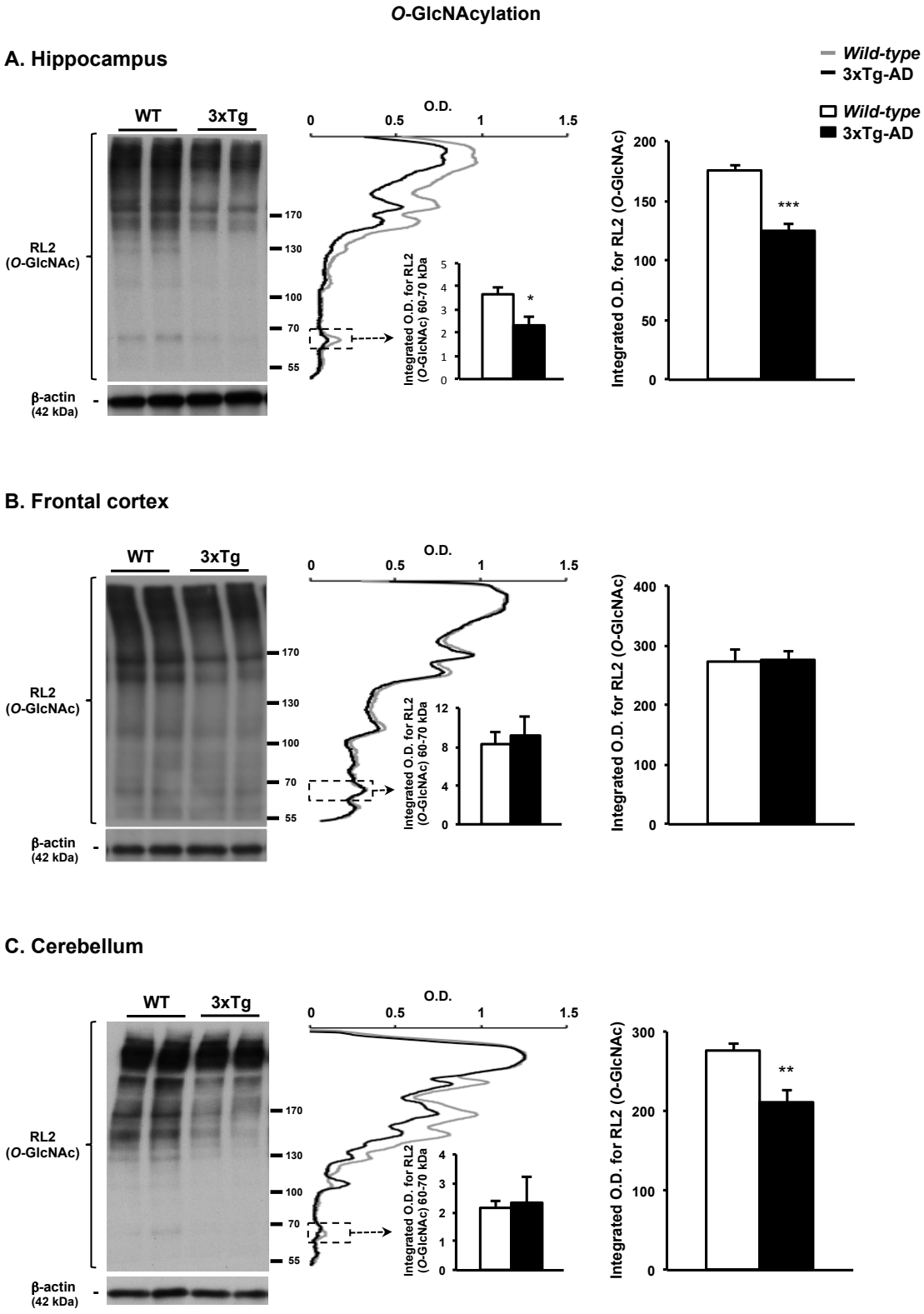

Figure 3 – Total *O*-GlcNAcylation levels are decreased in the hippocampus and cerebellum of 3xTg-AD mice. Western Blot analysis was performed in the hippocampus (A), frontal cortex (B) and cerebellum (C) of *wild-type* (WT) and 3xTg-AD mice. *O*-GlcNAcylation representative immunoblots are shown. *O*-GlcNAcylation immunoblot signals obtained with an RL2 antibody are analyzed in a spectral mode with the optical density (O.D.) on β-actin ratio as a function of the molecular weight. Total signal as well as the 60-70 kDa bands are presented as histograms. Values are means ± S.E.M. of 5 determinations per group. $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***) vs. the respective *wild-type* mice.

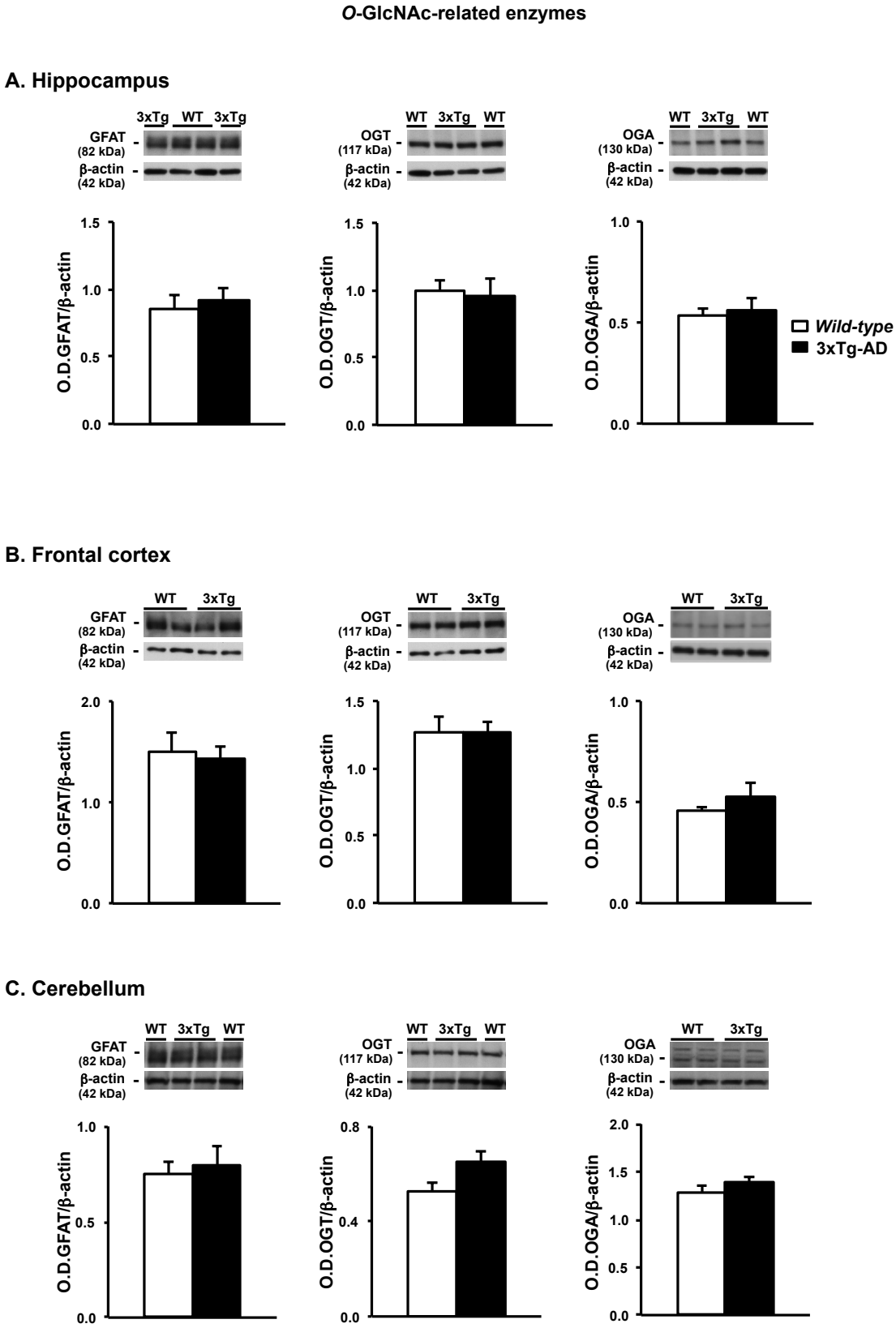

Figure 4 – Expression of *O*-GlcNAc-related enzyme is unchanged in 3xTg-AD mice. Western Blot analysis was performed in the hippocampus (A), frontal cortex (B) and cerebellum (C) of *wild-type* (WT) and 3xTg-AD mice. Representative immunoblots are shown for glutamine fructose-6-phosphate amidotransferase (GFAT), *O*-linked β-N-acetylglucosamine transferase (OGT) and *O*-GlcNAc hydrolase (OGA). Values are means ± S.E.M. of 5 determinations per group.

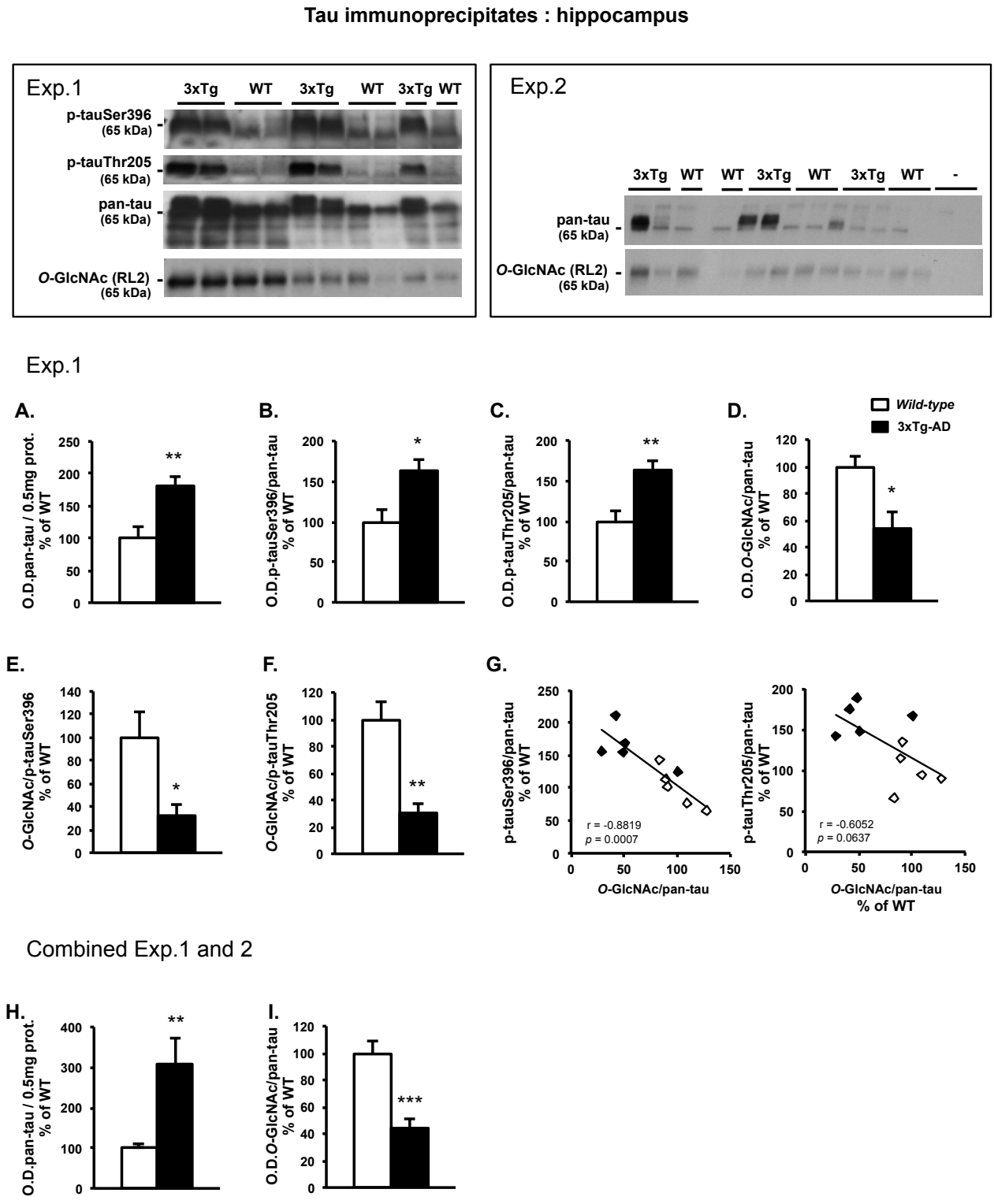

Figure 5 – Reduced *O*-GlcNAcylation of immunoprecipitated tau in the hippocampus of 3xTg-AD mice. p-tauSer396 and p-tauThr205 were obtained exclusively from experiment 1 (n=5 determinations per group) and normalized by the respective pan-tau values (B and C), whereas *O*-GlcNAc levels were obtained from experiments 1 (D) and combined experiments 1 and 2 (n=11 determinations per group) and also normalized by the respective pan-tau levels (I). Normalization of *O*-GlcNAcylated tau by p-tauSer396 (E) or p-tauThr205 (F), as well as correlation analyses between GlcNAcylated tau x p-tauSer396 and *O* -GlcNAcylated tau x p-tauThr205 (G) were performed using exclusively values from

experiment 1. Values in A-F, H and I are expressed as percent of *wild-type* mice and are means ± S.E.M. $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***) vs. the respective *wild-type* mice (Student's t test). The last two lanes of the blot in experiment 2 are from a pool of immunodepleted samples obtained for *wild-type* and 3xTg-AD mice.

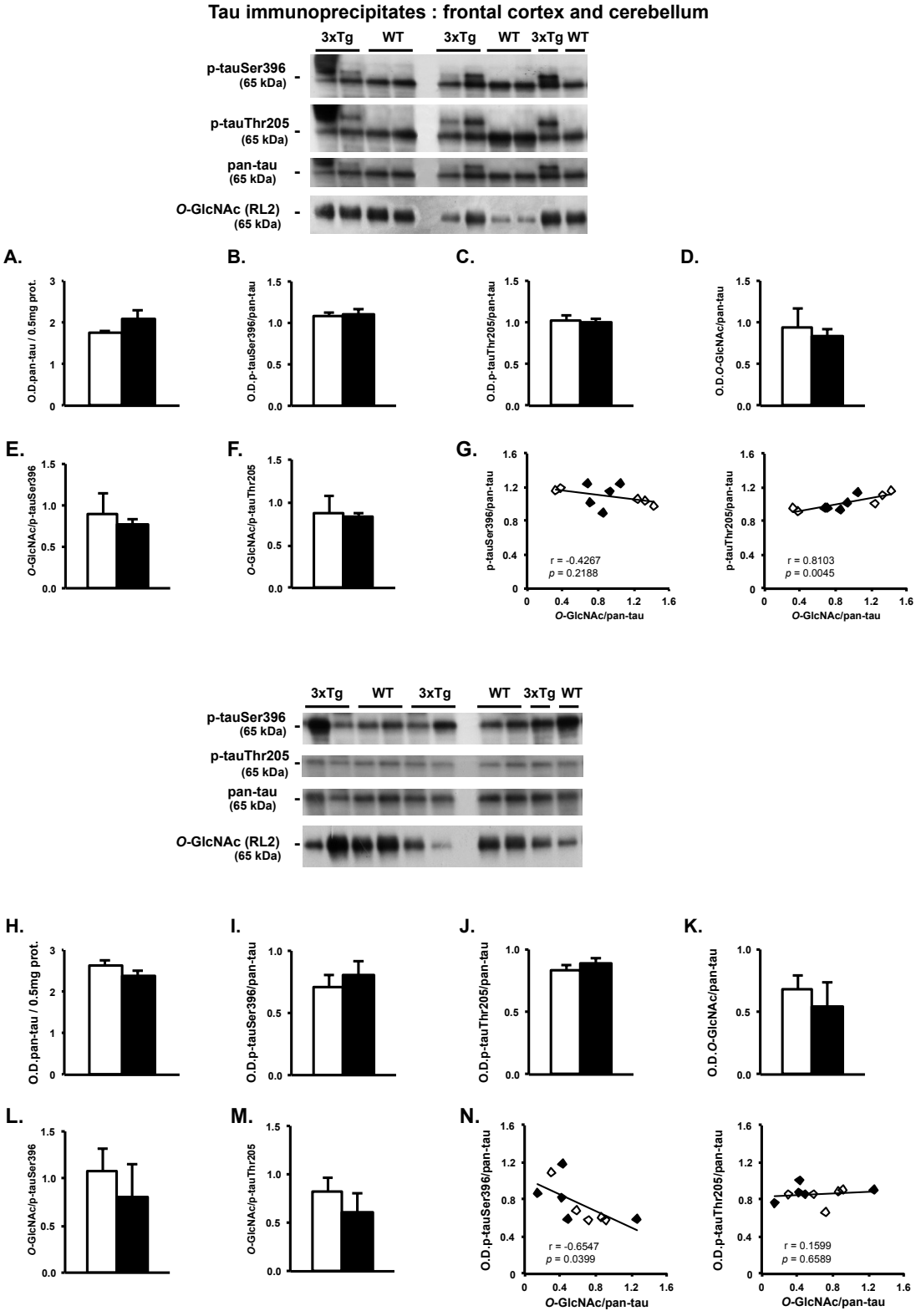

Figure 6 – *O*-GlcNAcylation of tau immunoprecipitated is unchanged in thefrontal cortex and cerebellum of 3xTg-AD mice. Western Blot analysis was performed in the frontal cortex and cerebellum of *wild-type* (WT) and 3xTg-AD mice. Immunoblots are shown for pan-tau, p-tauSer396, p-tauThr205, and *O*-GlcNAc. Histograms obtained for pan-tau (A, H), p-tauSer396 (B, I), p-tauThr205 (C, J), and *O*-GlcNAc (D, K), as well as ratio *O*-GlcNAc / p-tauSer396 (E, L) and *O*-GlcNAc / p-tauThr205 (F, M). Correlation found for *O*-GlcNAcylated tau x p-tauSer396 and *O*-GlcNAcylated tau x p-tauThr205 are shown (G, N). Values are means ± S.E.M. of 5 determinations per group.

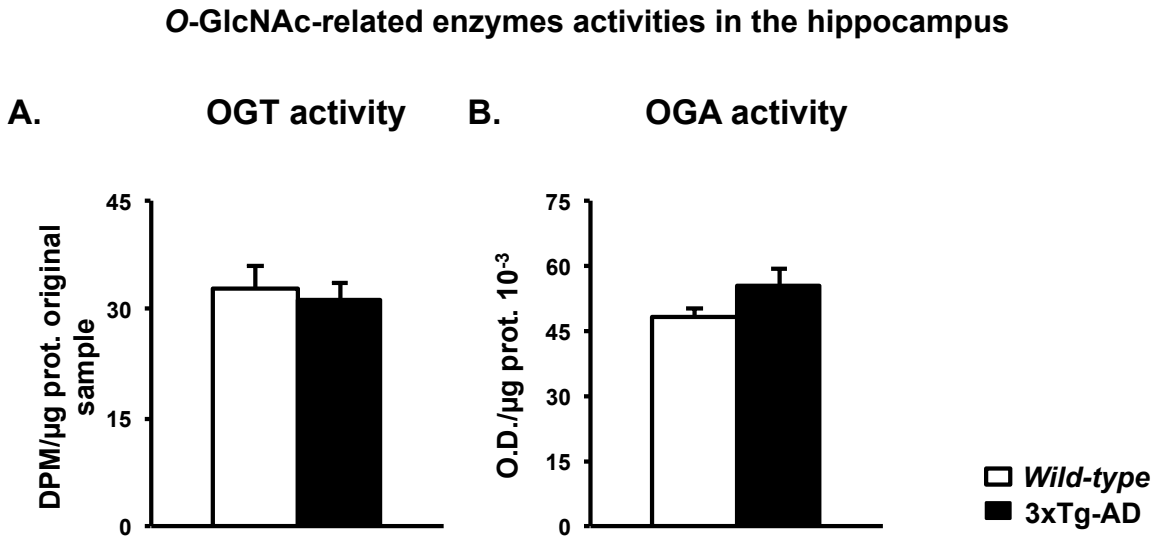

Figure 7 – OGT and OGA activities are not modified in the hippocampus of 3xTg-AD mice
Enzymatic activity was assessed in the hippocampus of *wild-type* (WT) and 3xTg-AD mice. OGT activity (A) was determined using a labeling with UDP-[^{3}H]GlcNAc. The ratio between DPM and the amount of protein of the original sample (µg) is presented. Values are means $\pm$ SEM of 5 determinations for 3xTg-AD mice and 3 for wild-type animals. OGA activity (B) was determined toward p-nitro-phenyl-N-acetyl-β-D-glucosaminide as a substrate. The ratio between optical density (O.D.) and amount of protein of the original sample (µg) is presented. Values are means $\pm$ SEM of 6 determinations per group.

CHAPTER TWO: Antidepressant-like effects of carbetocin on the pathological phenotype induced by early-life stress in adulthood

The World Health Organization (WHO) has ranked depression to the 4th place in pathologies contributing to disability worldwide (Kessler and Bromet, 2014). In 2012, more then 350 millions people of all ages were reported suffering from depression (WHO, 2012). Major depression is a very common human disorder, which involves several symptoms that occur together thus changing previous functioning and reducing the quality of life of the individual. The diagnosis of major depressive symptoms requires identification of specific symptoms that occur simultaneously for at least a 2-week period. The major features of depression are (*i*) subjective indication for depressed mood, (*ii*) reduced interest and pleasure (anhedonia) in all activities, (*iii*) variation in weight, (*iv*) sleep disturbances, (*v*) psychomotor agitation or retardation, (*vi*) loss of energy, (*vii*) reduction in concentration abilities, (*viii)* feelings of worthlessnss and inappropriate guilt and (*ix*) recurrent suicidal thoughts (DSM 5 criteria, American Psychiatric Association, 2013). In addition, important comorbidity has been found with anxiety (Zimmermann et al., 2014) and cognitive disorders (Goeldner et al., 2013).

Environmental factors as stressful life events might particularly be involved in depressive disorders (Kessler, 1997). Indeed, HPA axis hyperactivity has been found in depressed patients (de Kloet, Joëls and Hoslboer, 2005). From a neurochemical stand-point, mood disorders have been first associated to reduced levels of serotonin and/or noradrenaline in the brain, leading to the so-called "monoaminergic hypothesis of depression" (Bunney and Davis, 1965; Schildkraut, 1995). Nevertheless, several line of evidence also report changes in glutamate levels in patients suffering from depression (Kim et al., 1982; Altamura et al., 1993; Mauri et al., 1998). A positive correlation between the plasma glutamate levels and the severity of the depressive symptoms has also been found in patients (Mitani et al., 2006). Thus, the last two decades were the time for the emergence of glutamatergic theories of neuropsychiatric illness (Sanacora, Treccani and Popoli, 2012).

As described in the introduction section, exposure to stress during crucial period of life is a major risk factor for the development of psychiatric disorders. In the CNS, the hippocampus is particularly vulnerable to stress factors, with the ventral portion being a key region for stress response (Fanselow and Dong, 2010).

Previous work from our group used a proteomic analysis for the identification of key biomarkers involved in the pathological phenotype induced by early-life stress. The study of

the hippocampal proteome of PRS rats revealed changes in the expression profile of number of protein among which some involved protein involved in the regulation of synaptic vesicles and energetic metabolism (Mairesse et al., 2011). This work laid the groundwork for the study of the influence of early-life stress on neurotransmission. In particular, a causal relationship was demonstrated between anxiety-like behavior and a reduced glutamate release in the ventral hippocampus of PRS rats using a cocktail of mGlu2/3 receptor antagonist (LY341495) and $GABA_B$ receptor antagonist (CGP52432) that induced an enhanced depolarization-evoked aspartate release in the ventral hippocampus (Marrocco et al., 2012). In this chapter, we investigated how pharmacological modulation of the glutamatergic neurotransmission, particularly in the ventral hippocampus, could correct the pathological phenotype induced by early-life stress.

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

The model of PRS rats is endowed with face, construct and predictive validity. Treatment with antidepressant drugs in the adulthood was able to reverse the behavioral and neurological abnormalities induced by PRS (Morley-Fletcher et al., 2011; Mairesse et al., 2013). In this section, we assessed whether the alteration in glutamatergic neurotransmission found in PRS adult rats (Marrocco et al., 2012) could be corrected by chronic treatment with classical antidepressant drugs as fluoxetine or with a novel antidepressant as agomelatine.

To specifically examine neurotransmitters release in the ventral hippocampus from PRS rats, we used a particular procedure to isolate synaptosomes in Percoll gradient (Dunkley, Jarvie and Robinson, 2008). Prepared synaptosomes were then used for an *in vitro* study of neurotransmitter release (Fig. 24).

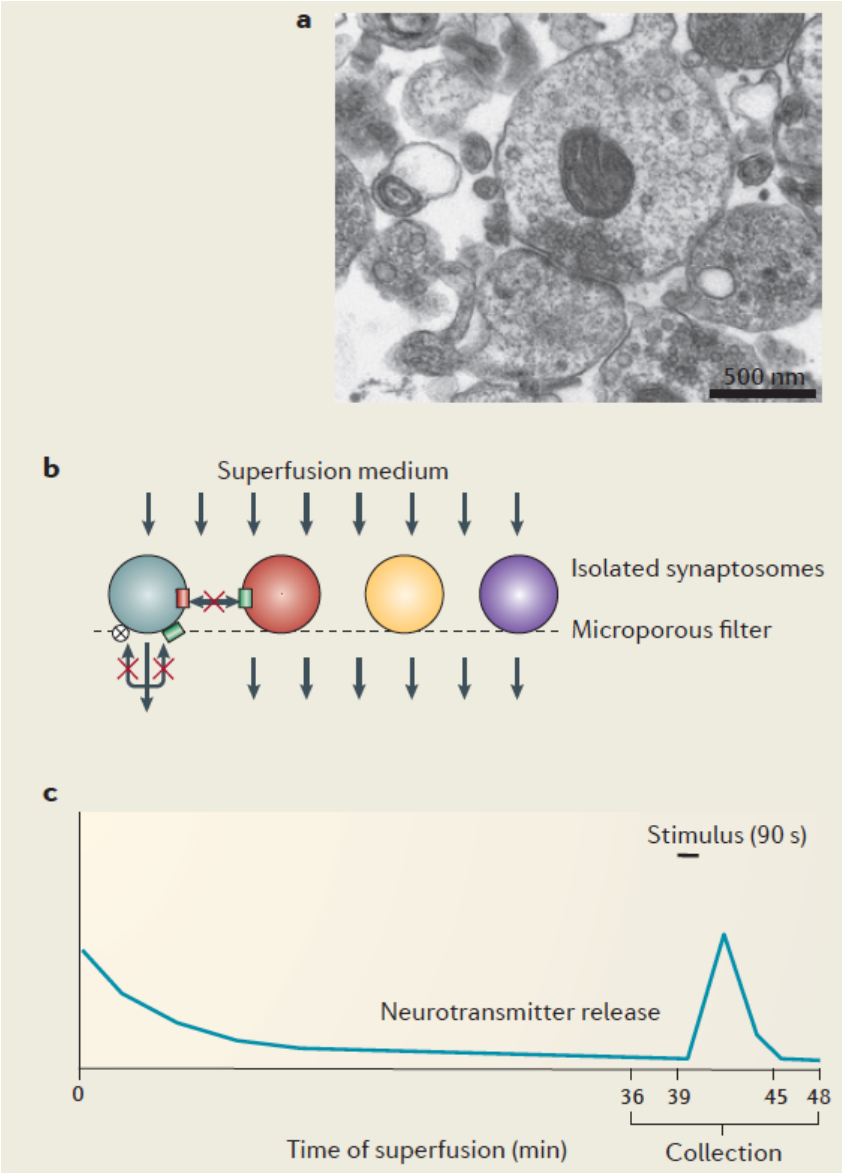

Figure 24: Measurement of endogenous neuroatransmitters release in superfused synaptosomes

The technique for measurement of neurotransmitter release from isolated synaptic terminals (synaptosomes) in superfusion was originally developed by Maurizio Raiteri and co-workers at the University of Genoa (Raiteri, Angelini and Levi, 1974). Release of a neurotransmitter elicits a chain reaction that ultimately results in a change in the release of that neurotransmitter (e.g. glutamate), as well as in the release of other neurotransmitters (such as serotonin, noradrenaline, and so on). This problem was solved by applying a thin layer of semi-purified or purified synaptosomes (a) on a microporous filter and applying a constant up–down superfusion to the sample (b). Through this method, any released endogenous transmitters and modulators are immediately removed by the superfusion medium before they can be taken up by transporters and activate autoreceptors or heteroreceptors on synaptic terminals. Reuptake can therefore not occur and indirect effects are minimized or prevented. In a typical experiment for measuring the release of endogenous amino acids such as glutamate or GABA, synaptosomes are layered in a thermostated superfusion and the collection of samples begins (c, see method section here below for further details; adapted from Popoli et al., 2012).

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Behavioral/Cognitive

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

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Abstract

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 characteristics of an anxious/depressive-like phenotype. Most neurochemical abnormalities of 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 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 days. 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 anxious-/depressive-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 (Ongür *et al.*, 2008; Tordera *et al.*, 2007; Garcia-Garcia *et al.*, 2009; Chen *et al.*, 2010; Popoli *et al.*, 2012; Musazzi *et al.,* 2010; 2013).

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 days 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 depression and anxiety, which has predictive, face and construct validity. A glutamatergic hypofunction is also found in *postmortem* brain tissues from depressed subjects (Choudary *et al.,* 2005; Bernard *et al.,* 2011). Of note, exposure to acute or chronic stress in the adult life results instead into an enhanced glutamate release in the hippocampus (Popoli *et al.*, 2012), 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 anxious/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 anxious/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 (SSRI), 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 demonstrates 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 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.

Methods

Animals. Forty nulliparous female Sprague-Dawley rats (20 for control and 20 for PRS groups), weighing approximately 250 g, were purchased from Charles River (France) 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 were taken from each litter for each measure to remove any litter effects (Becker and Kowall, 1977; Chapman and Stern, 1979). All experiments followed the rules of the European Communities Council Directive of September, 22nd, 2010, n°2010/63/EU. The local ethical committee approved the prenatal stress procedure.

Antidepressant treatment. Antidepressant drugs were dissolved in hydroxyethylcellulose (HEC 1% suspension in distilled water). Rats were 3 month-old at the beginning of the treatment. Control and PRS rats were treated daily during three weeks with i.p. injections of fluoxetine (5 mg/ml/kg, Sigma), agomelatine (40 mg/2ml/kg, Servier, France) or 1 ml/kg of HEC alone (vehicle). The dose of agomelatine was selected on the basis of previous reports (Van Reeth *et al.,* 1997; Papp *et al.,* 2003; Banasr *et al.,* 2006; Soumier *et al.,* 2009) and 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 and col. (1996). Injections were performed 2 h prior to the onset of the dark phase of the 12-h light/dark cycle, based on the circadian rhythm resynchronization properties and antidepressant activity of agomelatine (Van Reeth *et al.,* 1997; Papp *et al.,* 2003).

All animals used for *ex vivo* measurements of neurotransmitter release and immunoblot analysis of protein expression have 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 Dunkley and coworkers (1986), with minor modifications. Briefly, the tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with TRIS (final concentration 0.01 M) using a glass Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged at 1000 x *g* for 5 min, to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll gradient (6%, 10% and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 x *g* for 5 min. The layer between 10% and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. The synaptosomal pellet was then resuspended in physiological medium (standard medium) having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10 mM, glucose, 10; pH 7.2-7.4. Synaptosomes were incubated for 15 min a 37 °C in a rotary water bath and superfused at 0.5 ml/min with standard physiological solution. When studying the release of neurotransmitter evoked by high concentrations of K^+, synaptosomes were transiently (90 s) exposed, at t = 39 min, to 12 mM 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 aminoacid content. Endogenous glutamate and GABA were measured by HPLC analysis after pre-column derivatization with *o*-phthalaldehyde and separation on a C_{18} reverse-phase chromatographic column (10 X 4.6 mm, 3µm; at 30° C; Chrompack, Middleburg, The Netherlands) coupled to fluorimetric detector (excitation wavelength, 350 nm; emission wavelength, 450 nm). Buffers and the gradient program were as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol, 80:20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20:80; solvent C, 0.1 M sodium acetate (pH 6.0)/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; isocratic flow, 2 min; 78% A and 22% B in 2 min; isocratic flow , 6 min; 66% A and 34% B in 3 min; 42% A and 58% B in 1 min; 100% B in 1 min; isocratic flow, 2 min; 100% C in 3 min; flow rate, 0.9 ml min^{-1}. Homoserine was used as internal standard. Synaptosomal protein contents were determined according to Bradford (1976). The amount of endogenous glutamate and GABA from synaptosomes in superfusate fractions was expressed as picomoles per milligram of protein (pmol x mg^{-1} 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, dorsal and ventral hippocampi were rapidly dissected and immediately 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 x g for 10 min and resulting supernatants were centrifuged at 10000 x g for 15 min. The pellet obtained from the second centrifugation was resuspended in 10 volumes of HEPES- buffered sucrose and then spin again at 10000 x g for 15 min. This pellet contained the crude synaptosomal fraction. To validate the purity of this synaptosomal fraction we used anti-histon H3, anti-β tubulin, anti-synapsin Ia/b in immunoblot analysis. BCA assay was used to determine protein concentration. Synaptosomes lysates were resuspended in laemli reducing buffer and 20 µg of each sample were first separated by electrophoresis on Criterion TGX 4-15% precast SDS-polyacrylamide 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 (sc-20780, 1:4000), rabbit polyclonal anti-synapsin IIa (sc-25538, 1:4000) and rabbit polyclonal anti-VAMP (synaptobrevin, sc-13992, 1:2000), all purchased from Santa Cruz Biotechnology; mouse monoclonal anti-rab3a (107111, 1:2000) and mouse monoclonal anti-Munc-18 (116011, 1:2000), purchased from Synaptic System. All primary antibodies were incubated overnight at 4°C. HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (purchased from Amersham) were used a dilution at 1:10000 and incubated for 1 hour at RT. Densitometric analysis was performed with Quantity One software (Bio-Rad) associated to a GS-800 scanner. The ratio of individual proteins to β-actin was then determined and these values were compared for statistical significance.

Behavioral analysis

Assessment of social memory

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 3 sessions (3 session/day), a given juvenile (handled with rubber gloves) was introduced into the home cage of the adult for 5 min in a normally illuminated, quiet room during the light phase of the cycle (i.e., between 13:30 h and 18:30 h). Then, the juvenile was removed, kept individually in a cage (39 x 24 x 16 cm) with fresh bedding and food and water *ad libitum* for a defined inter-exposure interval (IEI) 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, Wageningen, The Netherlands).

Assessment of anxiety-like behaviour

Light and dark box. 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 two compartments: one light compartment (45 X 32 X 32 cm, 50 lux; light box) and one dark compartment (30 X 32 X 32 cm, 5 lux). The compartments were connected *via* a small opening (10 X 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 online *via* a video camera located above the box. Behavior was automatically analyzed using video tracking software (View Point, France).

Assessment of depressive-like behaviour

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 carried out between 12:00 and 17:00 h. Twenty-four hours after a 15-min session (pre-test, on day 15), control and PRS rats were tested (day 16) for a 5-min session during which immobility latency and duration, climbing, and swimming were automatically analyzed using a video tracking software (View Point, France).

Splash test. At day 19 of treatment a separate set of animals (n= 6-8 per group) were submitted to 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 minutes 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 with 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).

Statistical analysis

Data were analyzed by two-way ANOVA (group by treatment) with the exception of data of social memory, which were analyzed by three-way ANOVA for repeated measures (group by treatment by 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 as 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 hippocampus of control and PRS rats. PRS rats treated with vehicle for 21 days showed significant reductions in the levels of synapsin Ia/b (group x treatment, $F_{(2,18)}$ =6.87, p<0.01), synapsin IIa (group x treatment, $F_{(2,18)}$ =4.25, p<0.05), VAMP (synaptobrevin) (group x treatment, $F_{(2,18)}$ =3.55, p=0.05), and Rab3A (group x treatment $F_{(2,18)}$ =3.54, p=0.05), and a trend to a reduction in munc18 as compared to control unstressed rats treated with vehicle (**Fig. 1**; see also Marrocco *et al.*, 2012). Chronic treatment with either agomelatine (40 mg/kg/day; i.p.) or fluoxetine (5 mg/kg/day; i.p.) for 21 days 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 auto/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 (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, as compared to the respective control group (ANOVA group x treatment, $F_{(2,24)}$ =18.67, p<0.01) (**Fig. 2A**). 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 to 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. 2B**).

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 towards a juvenile as a test for social memory (Dantzer *et al.*, 1987). The same groups of animals used for measurements of glutamate release were tested for social memory at day 15, anxiety-like behavior 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 x treatment $F_{(2,48)}$ =4.95, p<0.05) (**Fig. 3A**). 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 x treatment, $F_{(2,48)}$ =4.24, p<0.05) (**Fig. 3B**). PRS rats showed a 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 x treatment $F_{(2,37)}$ =4.49, p<0.05) (**Fig. 3C**). 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 x treatment x 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.

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, depressive-like behavior in the forced-swim test and the social memory (see above). We examined, for each animal in each group, the correlation between depolarization-evoked glutamate release in the ventral hippocampus and (*i*) the latency to enter the light box; (*ii*) the immobility time in the forced-swim test; and (*iii*) the reduction in sniffing behavior at the 2nd 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 towards 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 *et al.,* 2013). The following factors might contribute to explain these contrasting findings: (*i*) the different breeding of the animals (reared from birth in the animal facility in our study); (*ii*) the execution of multiple behavioral tasks prior to the assessment of glutamate release in our study; (*iii*) ventral *vs.* total hippocampus in the two studies; and (*iv*) the different concentrations of K^+ ions used to stimulate glutamate release (12 *vs.*15 mM) resulting into a different extent 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 (reviewed by Maeng and Zarate, 2007). To date, the effects of classical 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, which recapitulate 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 (see 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 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 (*i*) agomelatine was highly effective in improving both anxiety-like behavior and social memory in PRS rats; and (*ii*) 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 provides the first pharmacological validation for the "glutamatergic hypothesis" of stress-related disorders (Holden *et al.*, 2003; Hashimoto *et al.*, 2009; Popoli *et al.*, 2012; McCarthy *et al.*, 2012) by demonstrating the action of novel and classical antidepressants in the PRS model, which replicates developmental factors involved in the etiology of anxious/depressive disorders (reviewed by Krishnan and Nestler, 2010), and has also 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 (mal)adaptive programming caused by early life stress, represents an attractive pharmacological target for the development of novel therapeutic strategies.

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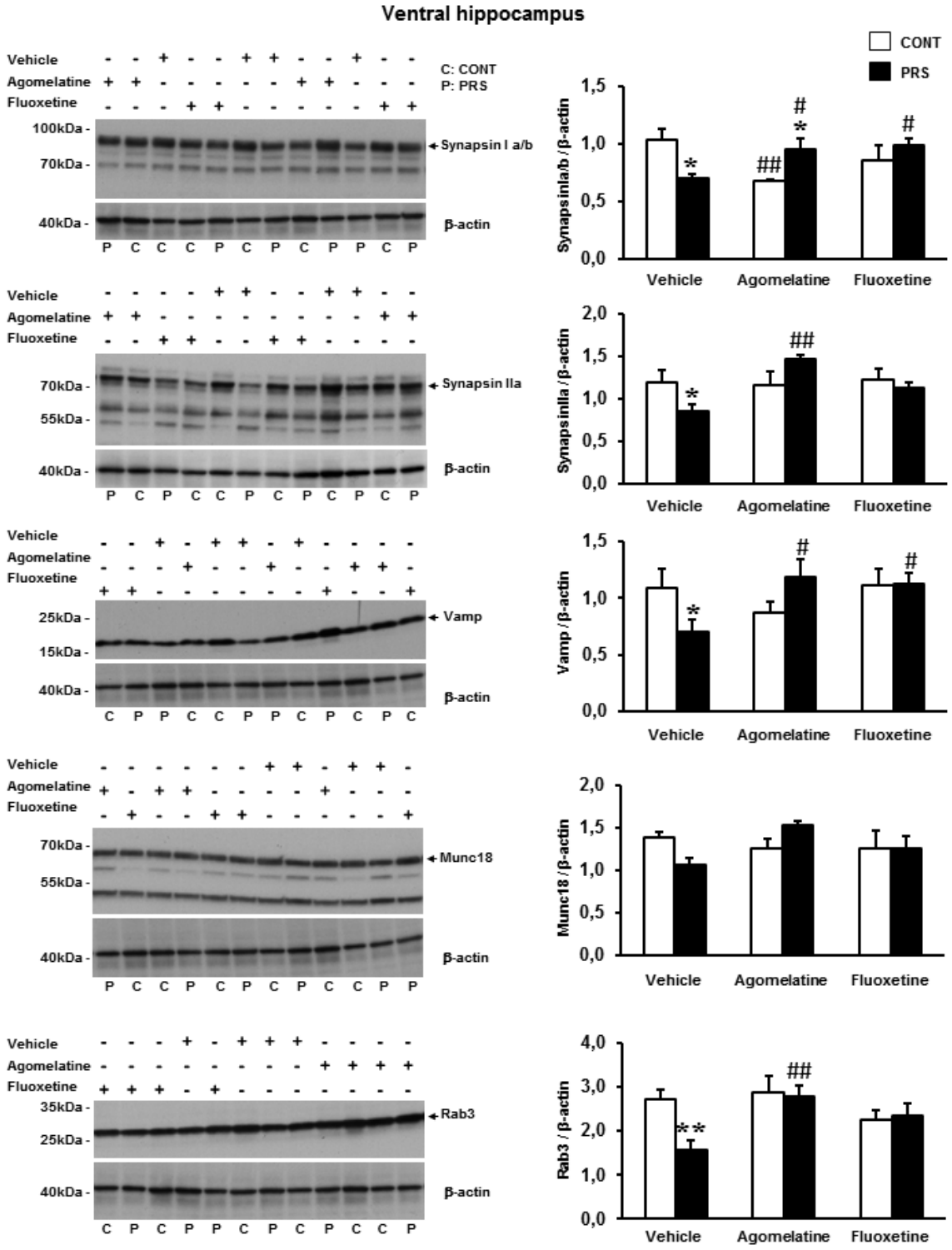

Figure 1: Chronic treatment with agomelatine or fluoxetine corrects abnormalities in the expression of synaptic vesicle-associated proteins in the ventral hippocampus of PRS rats.
Control unstressed rats (CONT) and PRS rats were treated i.p. with either vehicle, agomelatine (40 mg/kg) or fluoxetine (5 mg/kg) for 21 days. Representative continuous, uncropped image of 12 samples immunoblots (2 samples per group per treatment) are shown on the left side. Values are means ± S.E.M. (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.

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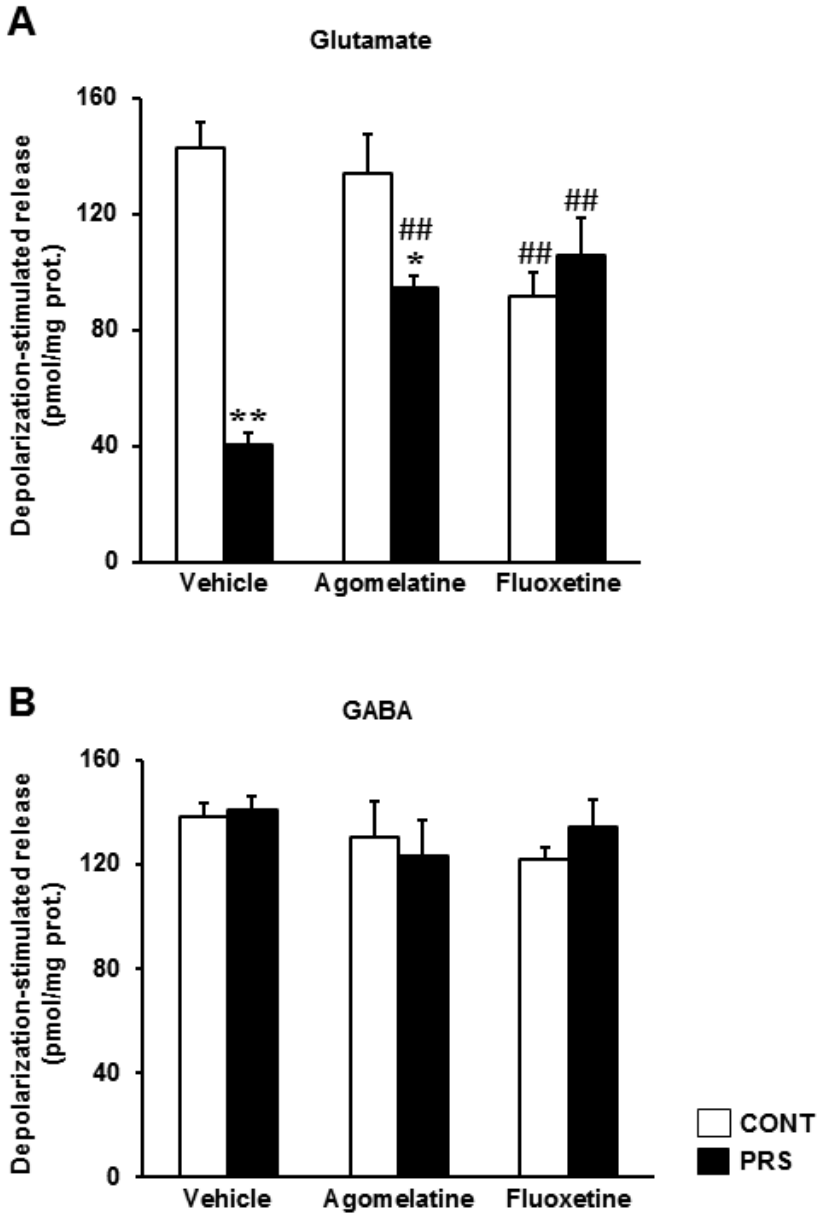

Figure 2: Chronic treatment with agomelatine or fluoxetine largely restores glutamate release in synaptosomes prepared from the ventral hippocampus of PRS rats.

Depolarization-evoked glutamate (A) or GABA (B) release was assessed in superfused synaptosomes prepared from control unstressed (CONT) and PRS rats treated with vehicle, agomelatine (40 mg/kg) or fluoxetine (5 mg/kg) for 21 days. Values are means ± S.E.M. (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.

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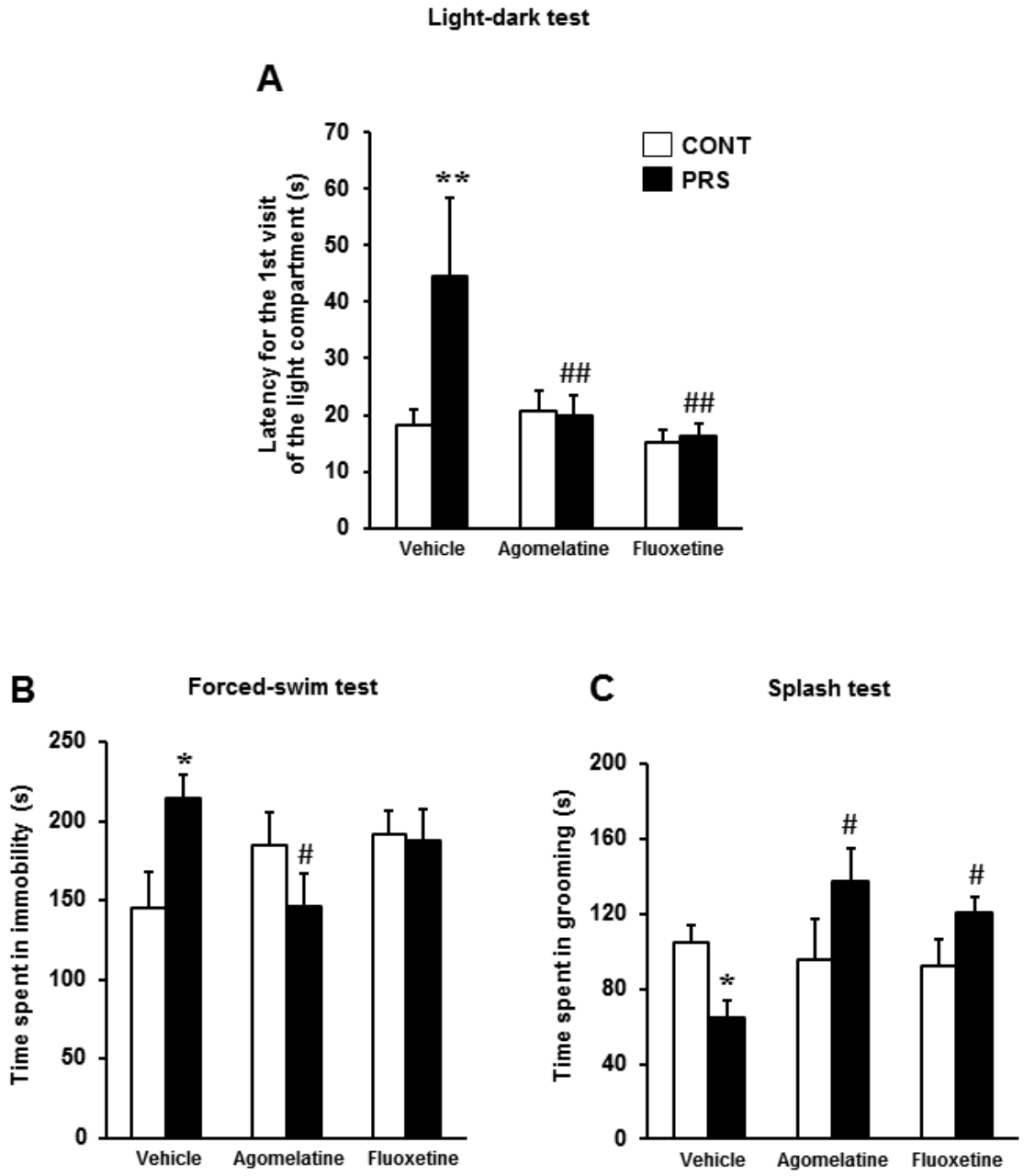

Figure 3: Chronic treatment with agomelatine or fluoxetine corrects anxiety- and depression-like behaviors in PRS rats.

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 i.p. with either vehicle, agomelatine (40 mg/kg) or fluoxetine (5 mg/kg) for 21 days. Values are means ± S.E.M. (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.

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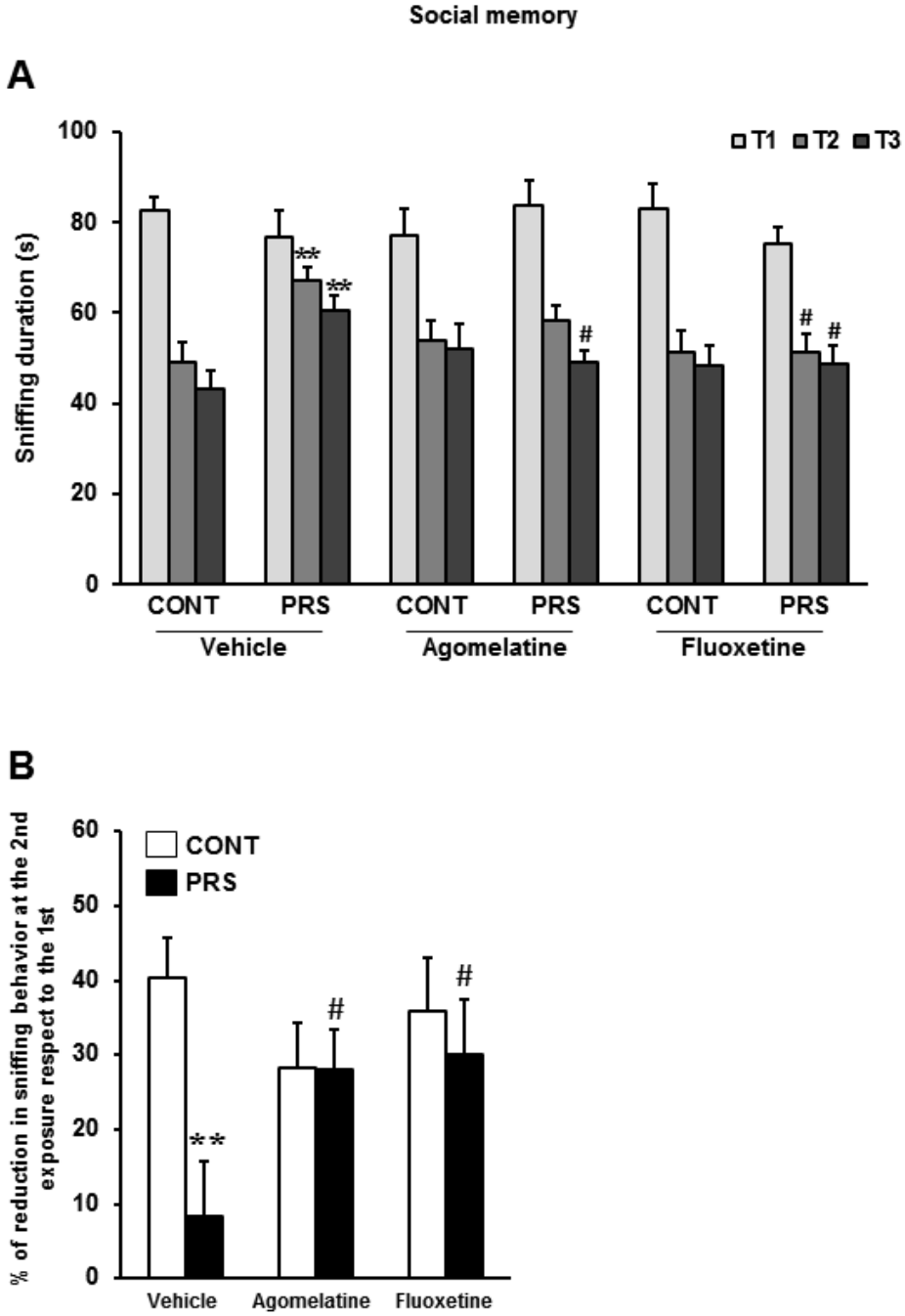

Figure 4: Chronic treatment with agomelatine or fluoxetine improves social memory in PRS rats. Control unstressed rats (CONT) and PRS rats were treated i.p. with either vehicle, agomelatine (40 mg/kg) or fluoxetine (5 mg/kg) for 21 days. Data of sniffing behavior at the first (T1), second (T2), and third (T3) exposure to the juvenile challenger are shown in (A). The percentage of reduction at the 2nd exposure respect to the first is shown in (B). Values are means ± S.E.M. (n=9 rats per group). $p<0.01$ (**) vs. the respective CONT rats; $p<0.05$ (#) vs. vehicle-treated CONT or PRS rats.

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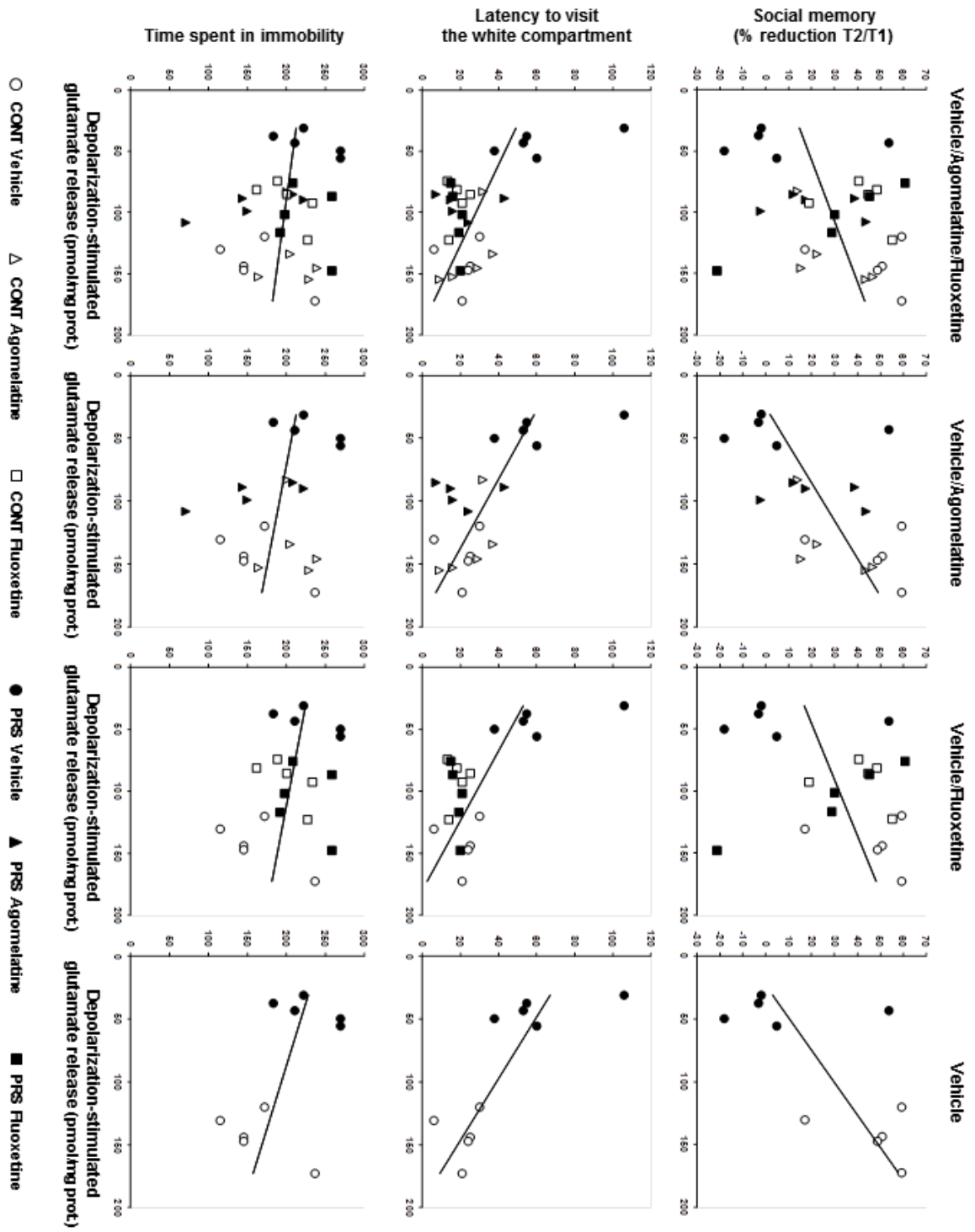

Figure 5: Correlation analysis of depolarization evoked glutamate release in the ventral hippocampus towards anxiety-like behavior in the light-dark box, depression-like behavior in the forced swim test, and social memory performance.

Pearson's correlation coefficient (*r*) and related *p* value are reported in Table 1 (n=5 rats per group).

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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.

Data represent Pearson's correlation coefficient (*r*) and related *p* value. The level of significance was set at p<0.05 (*).

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

2. Activation of presynaptic oxytocin receptors enhances glutamate release in the ventral hippocampus of prenatally restraint stressed rats.

In the previous section, we have seen that antidepressant drugs were able to correct early-life stress-induced behavioral and neurochemical abnormalities in the adulthood. Here, we investigated whether stimulation of endogenous endocrine systems could reverse de developmental trajectory induced by perinatal stress. Oxytocin is a neurohypophyseal hormone mainly known for its ecbolic activity during labor and its role in lactation. However, the function of oxytocin goes far beyond the peripheral regulation of reproduction, and central effects of oxytocin have been the subject of extensive investigation since the discovery that peripheral or intrahippocampal injection of oxytocin influences learning and memory processes (de Wied, 1965; Kovàcs et al., 1979). Over the past years, the role of hypothalamic peptides in the etiology of stress-related disorders has been largely investigated. As described previously, stress-induced central release of oxytocin plays an active role in HPA axis regulation (Engelmann, Landgraf and Wotjak, 2004). Oxytocin has prominent effects on social behavior and anxiety (De Dreu et al., 2010; Rotzinger, Lovejoy and Tan, 2010). Accordingly, anxyolitic properties of this nanopeptide have been demonstrated in rodents (McCarthy et al., 1996; Windel et al., 1997). The crucial role of the oxytocinergic system in the stress reponse, has also been shown in a recent work from Babic and colleagues (2015) indicating that pharmacological blockade of the oxytocinergic system with atosiban is able to modulate stress-induced neuroendocrine response to stress. Likewise, Lee and colleagues (2015) further demonstrate de neuroptrotective action of oxytocin in the regulation of stress-induced cognitive alterions. Interestingly, oxytocin also has antidepressant function (Arletti and Bertolini, 1987). The implication of oxytocin in stress-related behaviors and endocrine response (Neuman and Landgraft, 2012) suggests a putative neuromodulatory action of this nanopeptide, particularly in brain regions involved in stress response and emotional processing such as the amygdala, ventral hippocampus, and nucleus accumbens (Stoop, 2012), where oxytocin fibers and receptors have been described (Gimpl and Fahrenholz, 2001).

Thus, targeting the oxytocinergic system has potential clinical applications in psychiatric disorders, such as schizophrenia, post-traumatic stress disorder, addiction, and autism (Bartz and Hollander, 2006; Andari et al., 2010; Olff et al., 2010; Guastella and MacLeod, 2012; Higashida et al., 2012; Modi and Young, 2012).

Moving from the evidence that there is a causal relationship between the reduction of glutamate release in the ventral hippocampus and anxiety-like behavior of PRS animals, but also that chronic treatment with antidepressants was able to correct changes in glutamatergic transmission, we investigated whether activation of the oxytocinergic system could be involved in the modulation of glutamatergic neurotranmission. We used a selective oxytocin receptor agonist, carbetocin, which crosses the bood-brain barrier (Dvorskà et al., 1992) and has longer elimination half-life than oxytocin (Cort et al., 1981; Viero et al., 2010).

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Activation of presynaptic oxytocin receptors enhances glutamate release in the ventral hippocampus of prenatally restraint stressed rats

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Running tittle: Oxytocin and prenatal restraint stress rat model

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Abstract

Oxytocin receptors are known to modulate synaptic transmission and network activity in the hippocampus, but their precise function has been only partially elucidated. Here, we have found that activation of presynaptic oxytocin receptor with the potent agonist, carbetocin, enhanced depolarization-evoked glutamate release in the ventral hippocampus with no effect on GABA release. This evidence paved the way for examining the effect of carbetocin treatment in "prenatally restraint stressed" (PRS) rats, i.e. the offspring of dams exposed to repeated episodes of restraint stress during pregnancy. Adult PRS rats exhibit an anxious-/depressive-like phenotype associated with an abnormal glucocorticoid feedback regulation of the hypothalamus-pituitary-adrenal (HPA) axis, and, remarkably, with a reduced depolarization-evoked glutamate release in the ventral hippocampus. Chronic systemic treatment with carbetocin (1 mg/kg, i.p., once a day for 2-3 weeks) in PRS rats corrected the defect in glutamate release, anxiety- and depressive-like behavior, and abnormalities in social behavior, in the HPA response to stress, and in the expression of stress-related genes in the hippocampus and amygdala. Of note, carbetocin treatment had no effect on these behavioral and neuroendocrine parameters in prenatally unstressed (control) rats, with the exception of a reduced expression of the oxytocin receptor gene in the amygdala. These findings disclose a novel function of oxytocin receptors in the hippocampus, and encourage the use of oxytocin receptor agonists in the treatment of stress-related psychiatric disorders in adult life.

Keywords: perinatal stress; carbetocin; anxiety; social behavior; glutamate; ventral hippocampus

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1. Introduction

The function of oxytocin in the CNS has been the subject of extensive investigation since the early discovery that this hormone influences learning and memory processes (De Wied, 1965). Oxytocin has prominent effects on social behavior and anxiety (Ferguson et al., 2001; Jin et al., 2007; De Dreu et al., 2010; Insel, 2010; Labuschagne et al., 2010), and has potential clinical applications in psychiatric disorders, such as schizophrenia, post-traumatic stress disorder, addiction, and autism (Bartz and Hollander, 2006; Andari et al., 2010; Olff et al., 2010). The prevailing view is that oxytocin treatment in humans improves many aspects of social cognition and behavior, including interpersonal trust (Kosfeld et al., 2005; De Dreu et al., 2011), generosity (Zak et al., 2007), social recognition memory (Rimmele et al., 2009), and emotional empathy (Hurlemann, et al., 2010). However, oxytocin may facilitate social interaction, even if detrimental, as in case of impeded trust and cooperation, or closeness in insecurely or anxiously attached individuals (Bartz et al., 2010; 2011). To explain these findings, it has been suggested that oxytocin mainly acts as an anti-stress hormone (Windle et al., 1997; 2004; Bartz et al., 2011). Oxytocinergic neurons are mainly found in the hypothalamus, whereas oxytocin receptors are widespread in the CNS, and are found in brain regions implicated in stress response and emotional processing such as the amygdala, ventral hippocampus, and nucleus accumbens (Stoop, 2012). In the hippocampus, oxytocin receptors are found predominantly in the soma and dendrites of GABAergic interneurons and their activation increases the firing of interneurons, thereby suppressing the activity of pyramidal neurons (Mühlethaler et al., 1984; Zaninetti and Raggenbass, 2000). In a recent manuscript, Owen and colleagues (2013) have demonstrated that activation of oxytocin receptors in the hippocampus enhances the activity of fast-spiking GABAergic interneurons, thus improving information processing. Oxytocin receptors are also found in hippocampal synaptosomes (Audigier and Barberis, 1985), but their function is still unknown.

Abnormalities in hippocampal synaptic transmission and plasticity lie at the core of the pathological phenotype induced by prenatal stress (Yaka et al., 2007; Mairesse et al., 2012; Marrocco et al., 2012; 2014). PRS (prenatally restraint stressed) rats, i.e. the offspring of dams exposed to multiple episodes of restraint stress during pregnancy, represent an experimental animal model of anxious-depressive disorder endowed with face, construct, and pharmacological validity. These rats display anxiety- and depressive-like behaviors and show an excessive glucocorticoid response to acute stress, which is indicative of a dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis caused by an impaired hippocampal

glucocorticoid negative feedback (Maccari et al., 1995; Darnaudéry and Maccari, 2008). All these effects are reversed by chronic antidepressant medication (Mairesse et al., 2013; Marrocco et al., 2014). Interestingly, PRS rats show a selective reduction in glutamate release in the ventral hippocampus, which is causally related to the anxious-/depressive-like phenotype of PRS rats. Chronic treatment with fluoxetine or agomelatine, which produced antidepressant and anxiolytic effects in PRS rats, also corrected the defect in glutamate release in the ventral hippocampus in these rats (Marrocco et al., 2014). In addition, intrahippocampal injection of a cocktail of two drugs that enhance glutamate release (e.g. the type 2/3 metabotropic glutamate receptor antagonist, LY341495, and the $GABA_B$ receptor antagonist, CGP52432) abolished anxiety-like behavior in PRS rats (Marrocco et al., 2012).

Thus, the phenotype of PRS rats is particularly appropriate for the study of novel anti-stress drugs with mechanisms of action based on the modulation of hippocampal glutamatergic transmission.

Here, we examined whether activation of presynaptic oxytocin receptors could modulate glutamate release in the ventral hippocampus and whether, as a consequence of this mechanism, it could correct the pathological phenotype of PRS rats. To activate oxytocin receptors we used carbetocin, a potent and selective oxytocin receptor agonist, which has longer elimination half-life than oxytocin (Cort et al., 1981), and is clinically used for the control of postpartum bleeding (Engstrøm et al., 1998). Carbetocin can cross the blood-brain barrier (Dvorskà et al., 1992), and is known to affect animal behavior after peripheral administration (Klenerova et al., 2009; 2010; Chaviaras et al., 2010; Mak et al., 2012).

2. Materials and Methods

1.1.Animals

Nulliparous female Sprague-Dawley rats weighing 250-260 g (Charles River, France) were individually housed with a sexually experienced male rat for mating. A positive vaginal smear (presence of spermatozoa) defined the day 0 of gestation.

Control dams (n=21) were left undisturbed throughout gestation whereas stressed dams (n=15) were subjected to repeated episodes of restraint stress in a transparent cylinder (7.5 cm diameter, 19 cm long) under a bright light for 45 min three times daily (at 9:00 A.M., 12:00 P.M., and 5:00 P.M.) from day 11 of pregnancy until delivery (Maccari et al., 1995). Animals were maintained on constant temperature (22 ± 2 °C) and 12 h light/dark cycle (light on 8:00 A.M.). 3-month-old male offspring from litters containing 10-14 pups with an equivalent number of males and females were used for the experiments. A maximum of two male pups were taken from each litter for each measure to remove any litter effects. If two pups were taken, one was included in the group treated with saline, and the other in the group treated with carbetocin. All tests took place between 9:00 A.M. and 1:00 P.M.

All experiments were approved by the Institutional Animal Care and Use Committee in accordance with the principles of laboratory animal care (European Communities Council Directive of 1986, 86/609/EEC) and following the Institute for Laboratory Animal Research ‘‘Guide for Care and Use of Laboratory Animals”.

2.2 Chronic carbetocin treatment

Carbetocin (1 mg/kg ≈ 1 μmol/kg, SP080756, Polypeptide group, France) or saline was chronically administrated to 3 month-old animals, i.p., 1 h before the light switch-off, following a standard protocol used for the study of antidepressant action in PRS rats (Marrocco et al., 2014). The dose and route of administration of carbetocin were selected on the basis of a previous report (Klenerova et al., 2009). In a first set of experiments rats were treated daily with saline or carbetocin for 21 days in order to start behavioral testing after 2 weeks of chronic treatment (n=8-9 per group). Animals were tested for social interaction on day 15, for anxiety-like behavior on day 17, and for depressive-like behavior on days 18 and 19. On day 22, rats were killed for measurements of glutamate and GABA release (n = 5 per group). In a second set of experiments, PRS and control rats (n = 8 per group) treated daily with saline or

carbetocin for 15 days were used for the measurements of plasma corticosterone levels in response to novelty stress on day 14 and sacrificed on day 16 for the measurement of mRNA levels (n = 5 per group) in the hippocampus, amygdala and hypothalamus.

2.3 Behavioral analysis

2.3.3 Social memory. Social memory was assessed by measuring the ability of the tested animal to recognized a juvenile challenger using a procedure adapted from Engelmann et al., 1995. Rats were individually placed in transparent cages (39 x 24 x 16 cm) for 5 min for habituation in a normally illuminated, quiet room during the light phase of the cycle (e.g., between 3:30 and 6:30 P.M.). A juvenile male rat (1 month-old) was presented to the tested adult rat for 3 consecutive sessions (5 min each). The second presentation of the juvenile occurs 30 min after the first and a third, 120 min after the first. Sessions were video-recorded and the times spent in sniffing (the tested animal sniffs the challenger's fur) and play (rearing and anterior paws interactions with the challenger) were measured by a trained observer with Observer 2.0 (Noldus, The Netherlands).

2.3.2 Light and dark box test. Anxiety-like behavior was assessed using the light and dark. This apparatus is made up of the following two compartments: one light compartment (45 x 32 x 32 cm; 50 lux; light box) and one dark compartment (30 x 32 x 32 cm; 5 lux). The tested animal can freely explore the two compartments thanks to a small opening (10 x 15 cm) connecting the two compartments. Rats were placed in the light compartment and the time spent in the white box as well as the latency (in sec) to the first re-entry into the white box during the 5 min test session were recorded *via* a video camera placed above the white box. All the parameters were measured by a trained observer with Observer 2.0 (Noldus, The Netherlands) .

2.3.3 Forced swim test. Depression-like behavior was assessed using the forced-swim test (see Marrocco et al., 2014) on days 18 (15-min pretest session) and 19 (5 min test session) of treatment. Latency and duration of immobility and activity (climbing and swimming) were automatically analyzed using a video tracking software (View Point, France).

2.4 Measurements of glutamate and GABA release in superfused synaptosomal preparation

Synaptosomes prepared from the ventral part of the hippocampus (dissected out as shown in Fig. 1A) were used for the study of depolarization-evoked [^{3}H]D-aspartate, glutamate, and GABA release.

In a first set of experiments, carbetocin was applied directly *in vitro* on purified synaptosomes. Synaptosomes were preloaded with [2,3-^{3}H]D-aspartate (20-50 nM; sp. act. 11.3 Ci/ mmol, PerkinElmer) and depolarization-evoked [^{3}H]D-aspartate release was determined following the procedure described by Marrocco et al., 2012 (see also below). Synaptosomes prepared from control or PRS rats (n = 10 for control rats in Fig. 2A; n = 5 for both control and PRS rats in Fig. 2B), and challenged with multiple concentrations of carbetocin. Synaptosomes prepared from control rats (n = 3, each experiment in triplicate) were also used for measurements of endogenous glutamate and GABA release in response to carbetocin (see Fig. 2C).

We also performed *ex vivo* experiments in which synaptosomes were prepared from control and PRS rats (n = 5 per group, Fig. 3) chronically treated i.p. with either saline or carbetocin (1 mg/kg) once a day for 3 weeks, and used for measurements of basal and depolarization-evoked glutamate and GABA release without further addition of carbetocin.

For all these experiments, synaptosomes were purified according to the procedure described by Marrocco et al. (2012). 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 33,500g 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; pH7.2–7.4. Synaptosomes were incubated for 15 min a 37°C in a rotary water bath with or without [^{3}H]D-aspartate. Identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel chambers in a Superfusion System (Ugo Basile, Italy) maintained at 37°C and superfused at 0.5 ml/min with standard physiological solution.

Synaptosomes were transiently (90 s) exposed, at t = 39 min, to 12 mM K^+ (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 used for radioactivity counting ([^{3}H]D-aspartate release) or for measurements of endogenous glutamate and GABA.

Endogenous glutamate and GABA were measured by HPLC analysis after precolumn derivatization with o-phthalaldehyde and separation on aC18 reverse-phase chromatographic column (10 x 4.6 mm, 3 µm; at 30°C; Chrompack) coupled to a fluorimetric detector (excitation wavelength, 350 nm; emission wavelength, 450 nm).

Radioactivity in each superfusate fraction was quantified by liquid scintillation. The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). The depolarization-induced overflow was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6 min fraction collected during and after the depolarization pulse (evoked release, b2).

2.5 Novelty stress and plasma corticosterone levels

Novelty stress consisted in placing the animal in an unknown transparent cylindrical plexiglas cage (30 cm diameter, 50 cm high) without sawdust and under a bright light for 30 min.

Plasma corticosterone levels were measured from blood samples (about 200 µ1) withdrawn from the tail vein on freely moving animals at t_0 and at 30, 75, and 120 min after the onset of the novelty stress. Corticosterone levels were determined in duplicate using a commercial ELISA kit (DEV9922, Demeditec Diagnostics GmbH, Germany). The intra- and inter-assay coefficients of variation were 4.3 % and 6.5 %, respectively. Integrated values were calculated from the areas under the curve of corticosterone levels (using the trapezoidal method from t_0 to t_{120}) and divided by 120 to be expressed in ng/ml/min.

2.6 Measurements of mRNA levels

Rats were killed by decapitation, hippocampus, amygdala and hypothalamus were rapidly dissected and kept frozen at -80°C. Dissection of the amygdala was performed from a coronal slice (approximatevely from Bregma -1.7 mm to -3.6 mm) using a 5 mm punch (see Fig. 1). RNA extraction was performed using the RNeasy Plus mini kit (Qiagen, France). Concentrations of RNA samples were measured using Nanodrop (ND-1000, Labtech, Germany), and quality verified by Rin (RNA Integrity Number; Bioanalyzer 2100, Agilent Technologies, France). Retrotranscription was carried out with High-Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, France). Transcript levels were measured by real-time PCR using TaqMan assays (Applied Biosystems, France), probes set for TaqMan real-time PCR were obtained from Applied Biosystems: oxytocin/neurophysin 1 prepropeptide (Oxt, Rn00564446_g1); Oxt receptor (OxtR, Rn00563503_m1); corticotropin releasing hormone (Crh, Rn01462137_m1); Crh receptor 1 (Crhr1, Rn00578611_m1); Crh receptor 2 (Crhr2, Rn00575617_m1); arginine vasopressin (Avp, Rn00690189_g1); Avp receptor 1A (Avpr1a, Rn00583910_m1); Avp receptor 1B (Avpr1b, Rn01490541_m1); hydroxysteroid 11-beta dehydrogenase 1 (Hsd11b1, Rn00567167_m1); hydroxysteroid 11-beta dehydrogenase 2 (Hsd11b2, Rn00492539_m1); glucocorticoid receptor (GR, Nr3c1, Rn00561369_m1); mineralocorticoid receptor (MR, Nr3c2, Rn00565562_m1). Transcript levels were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn01775763_g1) expression. In order to assess the validity of the assay, we always included appropriate negative controls, in which cDNA had been omitted. These negative controls were undetermined by software with cycle thresholds >39.5. Acquisition of data was performed by means of StepOnePlus™ software, and data were obtained as C_t (threshold cycle). Then, a ΔC_t (C_t of the considered gene - C_t of the GAPDH gene) and a $\Delta\Delta C_t$ (C_t of a particular gene - C_t of the same gene in unstressed rats treated with saline) were calculated. Identical data were obtained when mRNA values were normalized by the expression of the ribosomal phosphoprotein (Rplp0, Rn00821065_g1).

2.7 Statistical analysis

Data were analyzed by one-way ANOVA (Fig. 2A), two-way ANOVA (Fig. 2B; 3A,B; 4B,C; 6B-E), or three-way ANOVA (Fig. 4A; 6A). The Newman-Keul's *post-hoc* test was used to isolate the differences. Student's t test was used for the analysis of data in Fig. 2C. Correlations were analyzed using the Pearson's correlation analysis. Significance was set to $p < 0.05$.

3. Results

3.1 Pharmacological activation of oxytocin receptors with carbetocin enhanced depolarization-evoked glutamate release in ventral hippocampal synaptosomes prepared from control or PRS rats

We assessed depolarization-evoked release of [^{3}H]D-aspartate (a non-metabolisable analogue of L-glutamate) in ventral hippocampal synaptosomes prepared from control rats and incubated in the presence of increasing concentrations of carbetocin (1, 3, 10, 30 or 100 nM). *In vitro* addition of carbetocin enhanced depolarization-evoked [^{3}H]D-aspartate release with an inverse U-shaped concentration-response curve. The effect of carbetocin was significant at 10 and 30 nM, and vanished at 100 nM (Fig. 2A). These data were confirmed in a second experiment carried out using 3 or 10 nM carbetocin, in which the analysis was extended to ventral hippocampal synaptosomes prepared from adult PRS rats. Under basal conditions (i.e., in the absence of carbetocin), depolarization evoked [^{3}H]D-aspartate release was significantly reduced in synaptosomes prepared from PRS rats, as expected (Marrocco et al., 2012). Interestingly, the concentration of 3 nM of carbetocin, which had no effect on depolarization-evoked [^{3}H]D-aspartate release in synaptosomes from control rats, significantly enhanced depolarization-evoked [^{3}H]D-aspartate release in PRS rats. In PRS synaptosomes, treatment with 3 or 10 nM carbetocin normalized depolarization-evoked [^{3}H]D-aspartate release (i.e., release was not different from that detected in untreated synaptosomes from control rats). However, there was still a significant difference in release between control and PRS synaptosomes treated with carbetocin (Fig. 2B). These experiments performed with [^{3}H]D-aspartate suggested that carbetocin was able to stimulate glutamate release from hippocampal synaptosomes, and that this effect was maintained in synaptosomes from PRS rats. However, the possibility exists that [^{3}H]D-aspartate is taken-up and released by GABAergic terminals, which are known to express the vesicular glutamate transporter (Noh et al., 2010). To exclude this possibility we measured depolarization-evoked release of endogenous glutamate and GABA in hippocampal synaptosomes of unstressed rats under basal conditions and in response to 30 nM carbetocin. Addition of carbetocin largely enhanced glutamate release but had no effect on GABA release (Fig. 2C).

We also examined whether chronic treatment with carbetocin in animals could induce sustained changes in glutamate release that could be visible in *ex vivo* experiments (i.e., in synaptosomal preparations), and was able to correct the reduction in depolarization-evoked glutamate release found in the ventral hippocampus of PRS rats (Marrocco et al., 2012). Control and PRS rats were treated i.p. with saline or carbetocin (1 mg/kg) once a day for 3 weeks, and depolarization-evoked glutamate or GABA release was measured in synaptosomes prepared from the ventral hippocampus without further addition of carbetocin. Chronic carbetocin had no effect on depolarization-evoked glutamate release in synaptosomes from control rats, but fully reversed the reduction in glutamate release observed in PRS rats treated with saline (Fig. 3A). Carbetocin treatment had no influence on depolarization-evoked GABA release in ventral hippocampal synaptosomes from either control or PRS rats (Fig. 3B).

3.2 Chronic carbetocin treatment reinforced social memory and reduced anxious/depressive-like symptoms in PRS rats

We performed behavioral studies in the same groups of rats used for the study of glutamate and GABA release. This allowed a correlation analysis between glutamate or GABA release and the selected behavioral tests. Control and PRS rats chronically treated with saline or carbetocin (1 mg/kg, i.p., daily for 3 weeks) were tested for (*i*) social interaction and social memory on day 15; (*ii*) anxiety-like behavior in the light-dark test on day 17; and (*iii*) depressive-like behavior in the forced-swim test on days 18 and 19 of treatment. Rats were killed 12-14 h after the last injection for the analysis of glutamate and GABA release in synaptosomes (see above).

When tested for social behavior, PRS rats showed a reduced paw-to-paw interaction ("play behavior") with a juvenile intruder during the first exposure, and a reduced ability to recognize the intruder (expressed as sniffing behavior) during the second and the third exposure (Fig. 4A). PRS rats also showed an increased anxiety-like behavior in the light-dark test (Fig. 4B), and an increased depressive-like behavior in the forced swim test (Fig. 4C). All these behavioral abnormalities were corrected by carbetocin treatment (see also Chavarias et al., 2010 for the antidepressant-life effect of carbetocin). Remarkably, carbetocin had no effect in any of the behavioral tests in control rats (Fig. 4A-C). Combining all data obtained in control and PRS rats regardless of the treatment, we found a positive correlation between the time spent in the light box and the reduction of sniffing behavior at the second exposure to the intruder (Fig. 4D). In contrast, there was no correlation between depressive like behavior and either social behavior (Fig. 4E) or anxiety-like behavior (Fig. 4F).

We extended the analysis to the correlation between behavior and *ex vivo* glutamate or GABA release from ventral hippocampal synaptosomes. Interestingly, we found a high and significant positive correlation between either social memory or anxiety-like behavior and glutamate release (Fig. 5A, B). No correlation was found between depressive-like behavior and glutamate release (Fig. 5C) or between behavioral data and GABA release (Fig. 5D-F).

3.3 Chronic carbetocin treatment normalized the glucocorticoid response to acute stress and corrected the abnormalities in the expression of stress hormone-related genes in PRS rats

These experiments were performed in additional groups of control and PRS rats treated with saline or carbetocin for 2 weeks. PRS rats are characterized by a prolonged glucocorticoid response to an acute stress (Maccari et al., 1995). Accordingly, PRS rats showed higher corticosterone levels 45 and 90 min after 30 min of exposure to a novelty stress (corresponding to 75 and 120 min after the beginning of the stress session) (Fig. 6A, B). Chronic carbetocin treatment (1 mg/kg, i.p., daily for 2 weeks) corrected the integrated levels of corticosterone in PRS rats (Fig. 6B). In control non-PRS rats, carbetocin treatment reduced peak corticosterone levels after novelty stressed without changing integrated corticosterone levels (Fig. 6A,B).

As biochemical correlates of the stress-prone phenotype of PRS rats, we measured the transcripts of oxytocin, oxytocin receptor, corticotropin-releasing hormone (CRH), types 1 and 2 CRH receptors, vasopressin, V1a and V1b vasopressin receptors (Avp), types 1 and 2 11β-hydroxysteroid dehydrogenase (11β-HSD1 and 2), glucocorticoid receptors (GR, Nr3c1), and mineralocorticoid receptors (MR, Nr3c2) in the hippocampus, amygdala and hypothalamus by real-time PCR. PRS rats showed a significant reduction in oxytocin, GR, MR, and 11β-HSD2 mRNA levels in the hippocampus, a large and significant reduction of oxytocin mRNA levels in the amygdala, and a significant increase in oxytocin receptor mRNA levels in the hippocampus and amygdala. All these changes were corrected by carbetocin treatment. Carbetocin also caused a large reduction of CRHR2 mRNA levels in the amygdala of control and PRS rats, which did not reach statistical significance with our post-hoc analysis (Fig. 6D).

4. Discussion

We could demonstrate for the first time that activation of presynaptic oxytocin receptors enhances depolarization-evoked glutamate release in the ventral hippocampus using an *in vitro* method that allows a clean estimation of a direct action of an exogenous compound on axon terminals without the confounding effect of cellular networks influencing neurotransmitter release, and without the bias of endogenous ligands acting at presynaptic receptors (Raiteri and Raiteri, 2000). We were surprised to find that pharmacological activation of oxytocin receptors with carbetocin was able to enhance glutamate release in hippocampal synaptosomes because oxytocin is believed to selectively activate GABAergic interneurons in the hippocampus (see Introduction and references therein). Thus, our data disclose a novel function of presynaptic oxytocin receptors (Audigier and Barberis, 1985), which might contribute to the modulation of hippocampal network activity by oxytocin (Owen et al., 2013). The direct and acute presynaptic action of carbetocin was even more pronounced in synaptosomes from PRS rats, in which [^{3}H]D-aspartate release was significantly enhanced by a concentration of the drug that was inactive in synaptosomes from control rats. This might be explained with the increased expression of oxytocin receptors found in the hippocampus of PRS rats, although we have no information on the receptor affinity for its ligand in these rats.

From a therapeutic standpoint, it was important to demonstrate that chronic carbetocin treatment could correct all “pathological” hallmarks of PRS rats we have tested, including the defect in glutamate release in the ventral hippocampus. One might argue that carbetocin could have been taken up during the treatment, and then released from purified synaptosomes thereby stimulating glutamate release. However, this is unlikely for the following reasons: (*i*) 12-14 hours elapsed from the last drug injection and the *ex vivo* preparation (the elimination half-life of carbetocin is less than 2 hours); (*ii*) the superfusion method minimizes the activity of compounds released from the tissue; and (*iii*) chronic carbetocin treatment had no effect on glutamate release in synaptosomes prepared from unstressed animals.

Thus, in PRS rats, chronic pharmacological activation of oxytocin receptors might have corrected some abnormalities in the molecular machinery controlling glutamate release, which are inherent to the phenotype of these rats. A first hypothesis is that these effects of chronic carbetocin treatment might be related to a direct action on glutamatergic terminals, as demonstrated when carbetocin was applied *in vitro* on purified synaptosomes. In fact, we previously demonstrated that the anxious-/depressive-like phenotype of PRS rats was causally

related to the selective reduction in glutamate release in the ventral hippocampus (Marrocco et al., 2012; 2014). As a consequence, the enhancement in depolarization-evoked glutamate release observed in the ventral hippocampus of PRS rats after chronic carbetocin treatment could sustain the anxiolytic and antidepressant properties of carbetocin in these rats. The increased activity of ventral hippocampal glutamatergic terminals could also be responsible for the restored HPA feedback mechanisms. Indeed, glutamatergic fibers projecting from the ventral subiculum to the paraventricular nucleus play a key role in in the feedback regulation of the HPA axis activity (Herman and Mueller, 2006). Moreover, the alterations of the HPA axis activity are in tight relation with the other phenotypic characteristics of PRS rats (for review Maccari and Morley-Fletcher, 2007). In other words, by restoring the glutamatergic transmission in the hippocampus of PRS rats, carbetocin could restore the central control of HPA axis feedback mechanisms and secondly normalize behavioral activity.

A second hypothesis is that the correction of the defect in glutamate release and related behavioral abnormalities by chronic carbetocin treatment occur downstream the normalization of the HPA axis activity in PRS animals. Indeed, chronic carbetocin treatment was able to normalize the expression of hippocampal mineralocorticoid (MRs) and glucocorticoids (GRs) receptors (Fig. 6C), which are involved in the negative feedback regulation of the HPA axis. The increased expression of MR and GR in the hippocampus of PRS rats after chronic carbetocin might have restored the efficiency of the negative feedback control of the HPA axis in PRS rats (see also Maccari et al., 1995) and helps to explain the normalization of the corticosterone response to a novelty stress. Along this line, hippocampal microinfusion of oxytocin was found to enhance MR expression and normalize the HPA response to stress in the predator scent stress (PSS) model of psychiatric disorders (Cohen et al., 2010). Also, a large body of evidence suggests that both MRs and GRs positively modulate excitatory neurotransmission and glutamate release in the hippocampus (Karst et al., 2005; Iqbal et al., 2006; Wang and Wang, 2009; Chatterjee and Sikdar, 2014; Treccani et al., 2014). Thus, the normalization of MR and GR expression associated with the normalization of the HPA activity in PRS rats could contribute to the normalization of the glutamatergic transmission in the ventral hippocampus associated with the reduced anxiety-like behavior and correlated improvement of social memory in these rats.

These two hypotheses are not exclusive, building up a complex scenario, in which stimulation of glutamate release and normalization of the HPA axis might be interconnected in a positive feedback loop that is regulated by oxytocin receptors. Chronic systemic carbetocin (present

data) or chronic i.c.v. oxytocin (Peters et al., 2014) might exert anti-stress and anxiolytic properties by activating this positive feedback loop perhaps at multiple levels. Signals from the amygdala may converge into this loop, and the large (albeit not significant) reduction in type-2 CRH receptor found in response to carbetocin in the amygdala might be relevant for the anti-stress effect of the drug. Of note, a tight interaction between type-2 CRH receptors and oxytocin has been repeatedly demonstrated (Arima and Aguilera, 2000; Dabrowska et al., 2011).

An interesting finding was that PRS caused opposite changes in the transcripts encoding for oxytocin and the oxytocin receptor both in the hippocampus and amygdala. PRS might have caused an oxytocinergic hypotonus (Lee et al., 2007; De Souza et al., 2012), with an ensuing up-regulation of oxytocin receptors in target regions of hypothalamic oxytocinergic neurons, i.e., the hippocampus and amygdala. Carbetocin treatment reduced oxytocin receptor mRNA levels in the amygdala and hippocampus of PRS rats, and in the amygdala of unstressed rats. This is in line with the evidence that continuous central infusion of oxytocin reduces oxytocin receptor binding in the lateral septum and amygdala (Peters et al., 2014), and suggests that expression of the oxytocin receptor gene is regulated by the extent of receptor activation. Changes in oxytocin transcript we have found in the hippocampus and amygdala are intriguing because in these structures intrinsic oxytocinergic neurons are not present (Ludwig and Leng, 2006), and oxytocinergic nerve terminals originate from the hypothalamus (Knobloch et al., 2012). It is possible that the oxytocin transcript detected in the amygdala and hippocampus is transported along the axon for a local synthesis of oxytocin in nerve terminals. PRS largely reduced oxytocin mRNA levels in the amygdala and hippocampus; but not in the hypothalamus, raising the possibility that early life stress might have affected mRNA trafficking. Our findings suggest that, in addition to the amygdala (Debiec, 2005; Lee et al., 2007; Choleris et al., 2008; Neumann, 2008; Veenema and Neumann, 2008), the ventral hippocampus is a target region for the anxiolytic and anti-stress activity of oxytocin. The ventral hippocampus is a key station of the stress neuronal circuit and is tightly connected to brain regions that are involved in the regulation of mood, anxiety, and social behavior, such as the ventral tegmental area, nucleus accumbens, amygdala, and medial prefrontal cortex (Belujon and Grace, 2011).

Chronic carbetocin treatment corrected all tested behavioral and neuroendocrine abnormalities of PRS rats, suggesting that pharmacological activation of oxytocin receptors blunts the expression of the pathological programming induced by early life stress. Interestingly, data of

anxiety-like behavior were correlated with data of social memory, suggesting a relationship between these behavioral paradigms. We have previously demonstrated a causal relationship between reduction in glutamate release in the ventral hippocampus and at least anxiety-like behavior and the defect in social interaction in PRS rats (Marrocco et al., 2012; 2014). We have confirmed this finding in the present paper with the observation of a high and significant positive correlation between either social memory or anxiety-like behavior and glutamate release. This raises the interesting possibility that the hippocampus might be a primary target for the behavioral effects of systemic carbetocin in our study. However, systemic carbetocin may act at multiple levels both in the periphery and the CNS and this could contribute to the behavioral effect of carbetocin in PRS rats.

In conclusion, our findings provide new insights into the complex and multifaceted action of oxytocin indicating that this action critically involves the regulation of glutamate release in the ventral hippocampus. In addition, our data support the use of oxytocin or oxytocin receptor agonists in the treatment of stress-related disorders that are related to glutamatergic dysfunction. The therapeutic potential of modulating the oxytocinergic system is strengthened by the evidence that carbetocin displayed a robust therapeutic activity in PRS rats, but had no effect in unstressed rats, therefore discriminating between physiological and pathological conditions. Developing a “disease-dependent” drug is an important goal in the treatment of psychiatric disorders.

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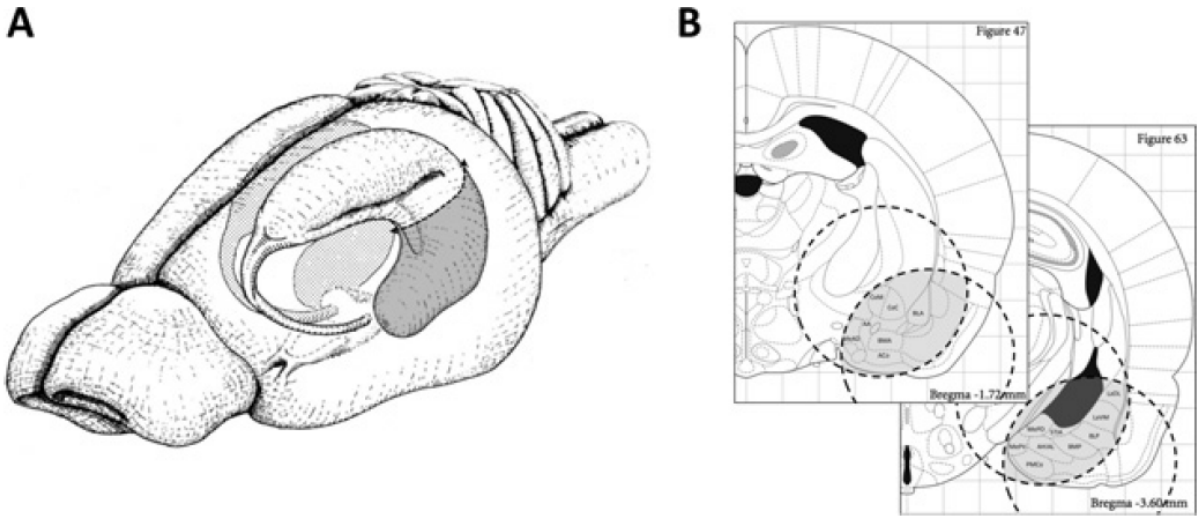

Figure 1: Dissection boundaries of ventral hippocampus and amygdala.
The ventral part of the hippocampus used for synaptosomes preparation was dissected following the boundary indicated by the grey area (A, adapted from Cheung and Cardinal, 2005). For taqman® real time PCR analyses, amygdala was taken off from a coronal slice (approximately from Bregma -1.7 mm to -3.6 mm) using a 5 mm punch (dotted line circles) allowing to keep the grey area including most of the amygdala nuclei (B, adapted from Paxinos and Watson, 2005).

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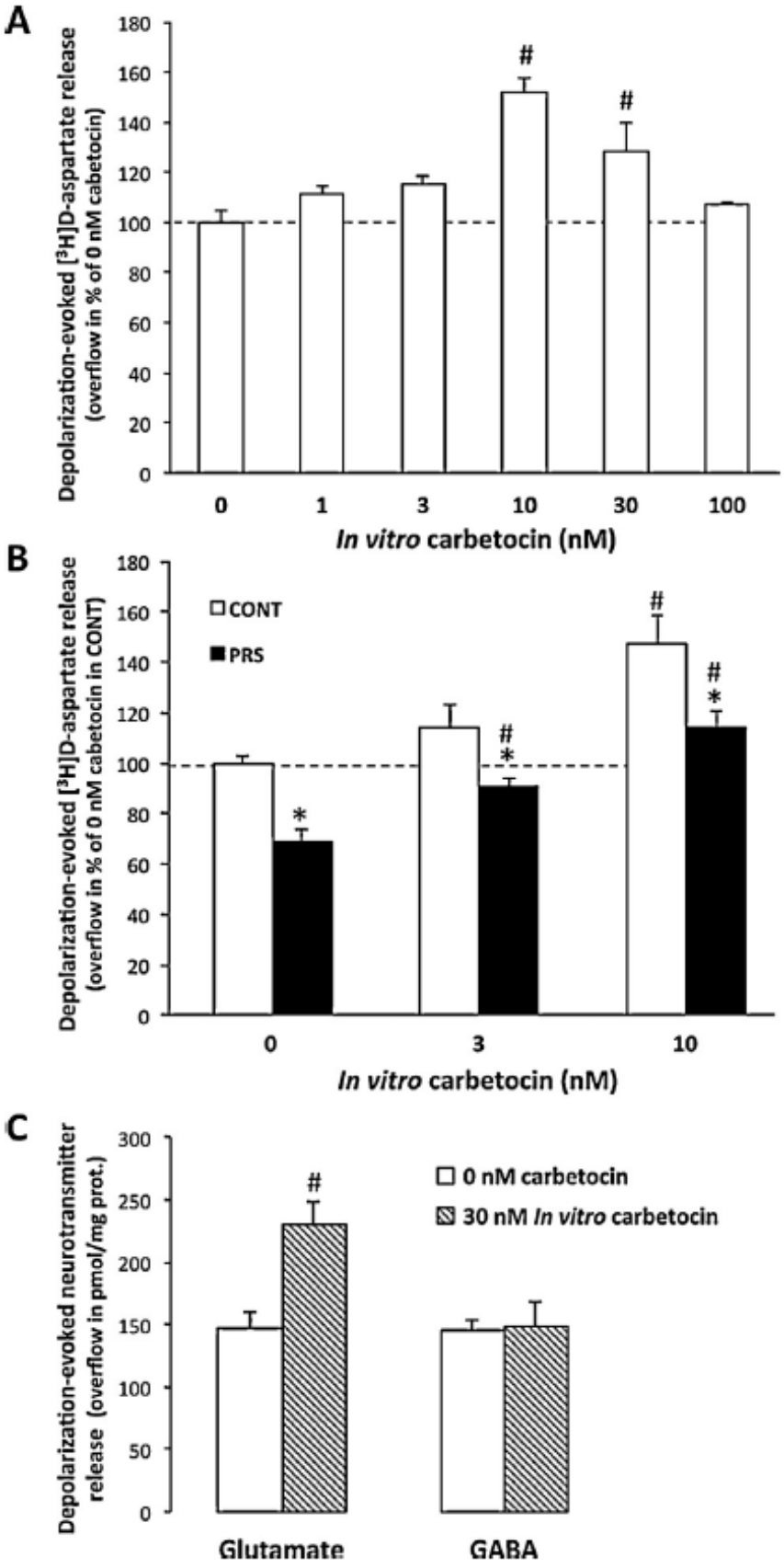

Figure 2: Carbetocin amplifies depolarization-evoked glutamate release in superfused synaptosomes prepared from the ventral hippocampus.

Release was assessed in superfused hippocampal synaptosomes under basal conditions or in response to depolarizing concentrations of KCl (12 mM). The effect of different concentrations of carbetocin on depolarization-evoked [^{3}H]D-aspartate release in synaptosomes prepared from control rats is shown in (A), where data are means ± S.E.M. of 6-10 determinations. # $p < 0.05$ vs. depolarization-evoked release in the absence of carbetocin ("0" value) (One-way ANOVA + Neumann-Keul's; $F_{5,35} = 61.3$ $p < 0.001$). The effect of carbetocin on depolarization-evoked [^{3}H]D-aspartate release in ventral hippocampal synaptosomes prepared from control or PRS rats is shown in (B), where values are means ± S.E.M. of 5 determinations. $p < 0.05$ vs. the corresponding values obtained in the absence of carbetocin (#), or vs. the respective values obtained in control (CONT) unstressed rats (*) (Two-way ANOVA + Neumann-Keul's; group effect $F_{1,8} = 26.7$ $p < 0.001$; treatment effect $F_{2,16} = 32.2$ $p < 0.0001$). The effect of carbetocin on depolarization-evoked glutamate and GABA release in ventral hippocampal synaptosomes from control rats is shown in (C), where values are means ± S.E.M. of 3 determinations (each in triplicate). # $p < 0.05$ vs. the respective values obtained in the absence of carbetocin (Student's t test; $t_{1,16} = -4.74$).

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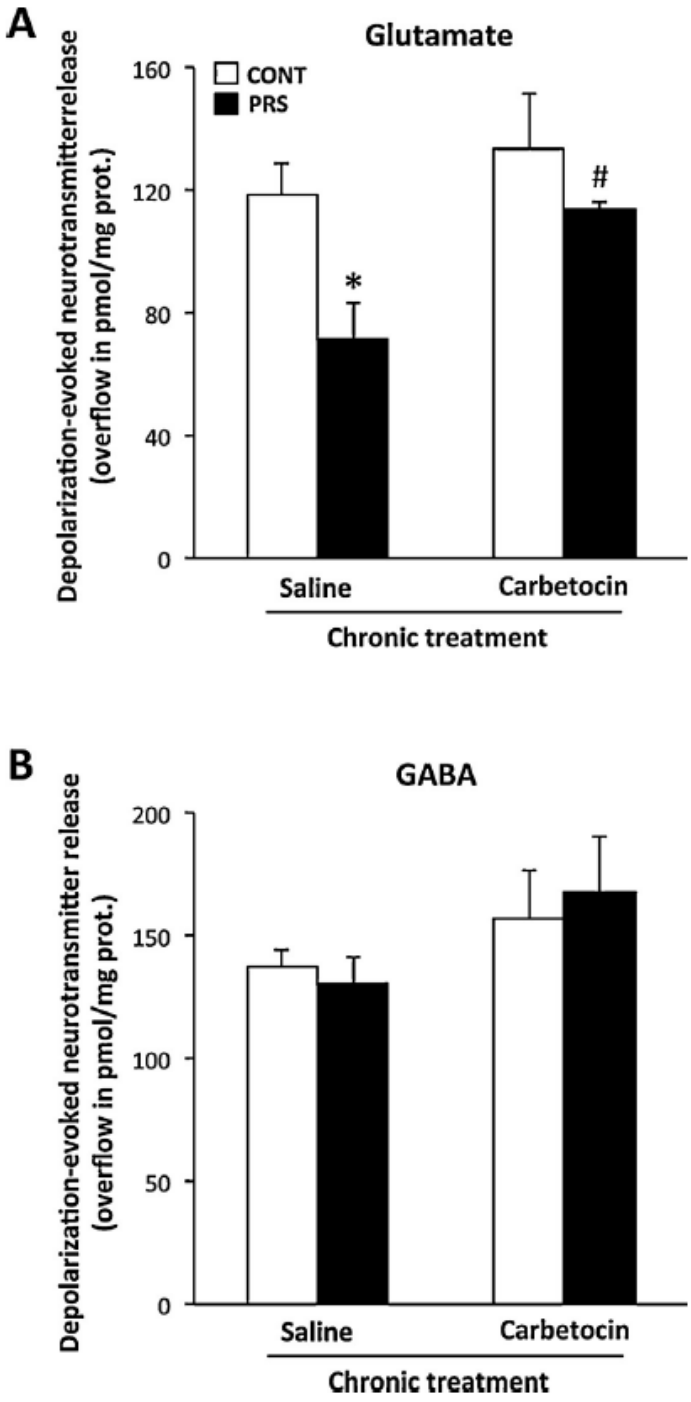

Figure 3: Chronic carbetocin treatment corrects the defect in depolarization-evoked glutamate release in ventral hippocampal synaptosomes from PRS rats.

The study was carried out with 5 of the 8 control (CONT) and PRS rats treated with saline or carbetocin (1 mg/kg) for 21 days and used for behavioral analysis. Depolarization-evoked glutamate and GABA release in ventral hippocampal synaptosomes is shown in (A) and (B), respectively (Two-way ANOVA: glutamate, group effect $F_{1\text{-}16} = 5.95$, $p < 0.05$; carbetocin effect $F_{1\text{-}16} = 4.42$, $p < 0.05$; GABA, *n.s.*). Data are means ± SEM of 5 determinations. Newman keuls post-hoc test: * $p < 0.05$, PRS *vs.* CONT; # $p < 0.05$, Carbetocin *vs.* saline.

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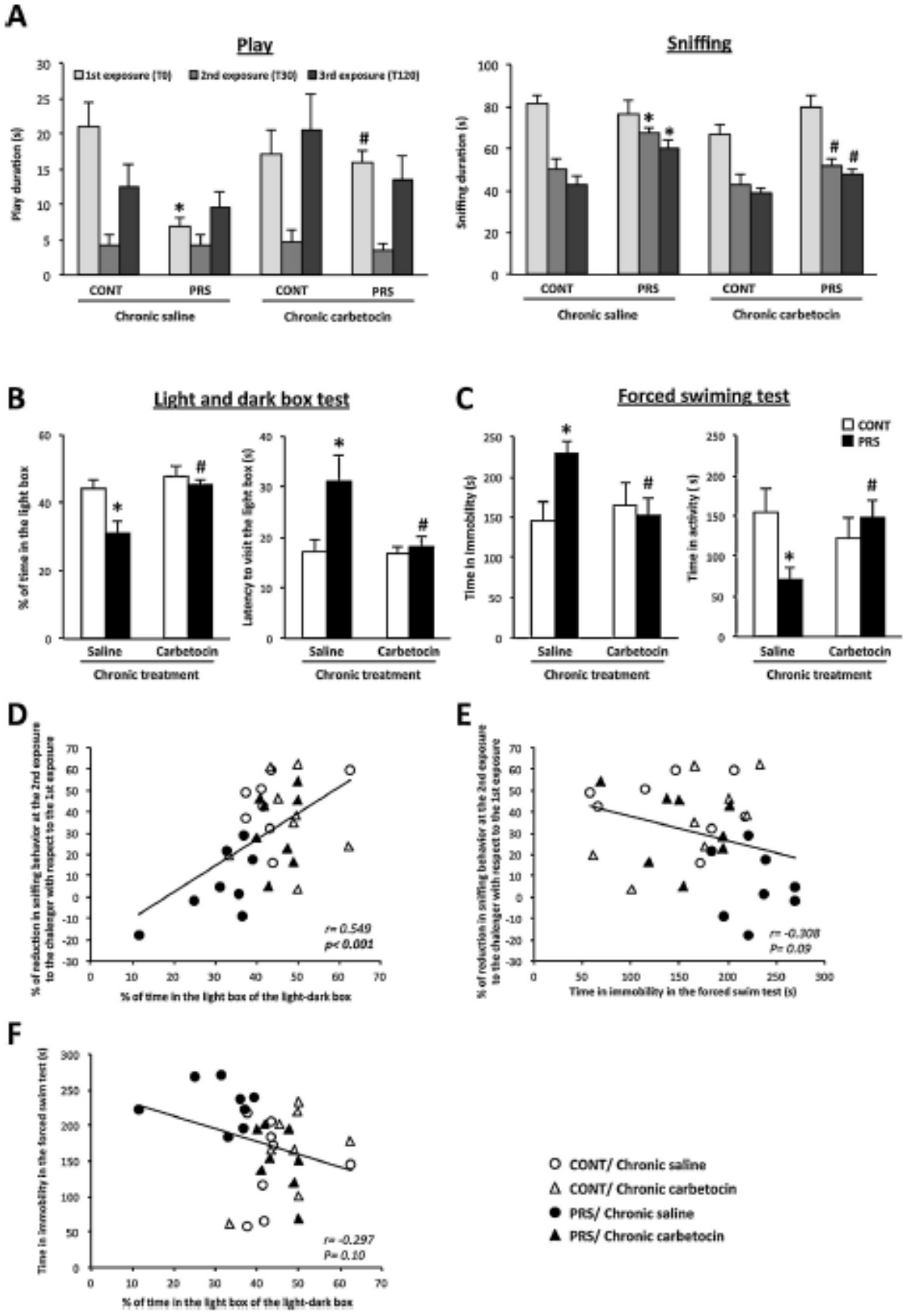

Figure 4: Chronic carbetocin treatment corrects anxiety-like and depressive-like behavior, and abnormalities in social behavior in PRS rats

Control (CONT) and PRS rats were treated i.p. with saline or carbetocin (1 mg/kg) daily for 21 days and tested for social behavior, anxiety-like behavior, and depressive-like behavior at day 15, 17, and 18/19 of treatment, respectively. Social behavior in response to repeated exposure to a juvenile rat is shown in (A). T30 and T120 correspond to 30 and 120 min following the first exposure, respectively (play behavior, group x treatment x time $F_{(2,64)} = 3.23$, $p < 0.05$; sniffing behavior, group x treatment x time $F_{(2,64)} = 3.28$, $p < 0.05$). Anxiety-like behavior in the light-dark box is shown in (B), (time in the light box, group x treatment $F_{(1,28)} = 4.1$, $p < 0.05$; latency to visit the light box, group x treatment $F_{(1,28)} = 4.5$, $p < 0.05$). Depressive-like behavior in the forced swim test is shown in (C) (immobility, group x treatment $F_{(1,28)} = 5.57$, $p < 0.05$; active behavior, group x treatment $F_{(1,28)} = 5.73$, $p < 0.05$). Values are means ± SEM of 8 determinations. Newman keuls post-hoc test: * $p < 0.05$, PRS *vs.* CONT; # $p < 0.05$, Carbetocin *vs.* saline. Correlations between social memory (% reduction of sniffing behavior at the second vs. the first exposure to the challenger) and anxiety-like behavior, social memory and depressive-like behavior, and anxiety-like behavior and depressive-like behavior are shown in (D), (E), and (F), respectively, with the r and *p* values of the Pearson's correlation test.

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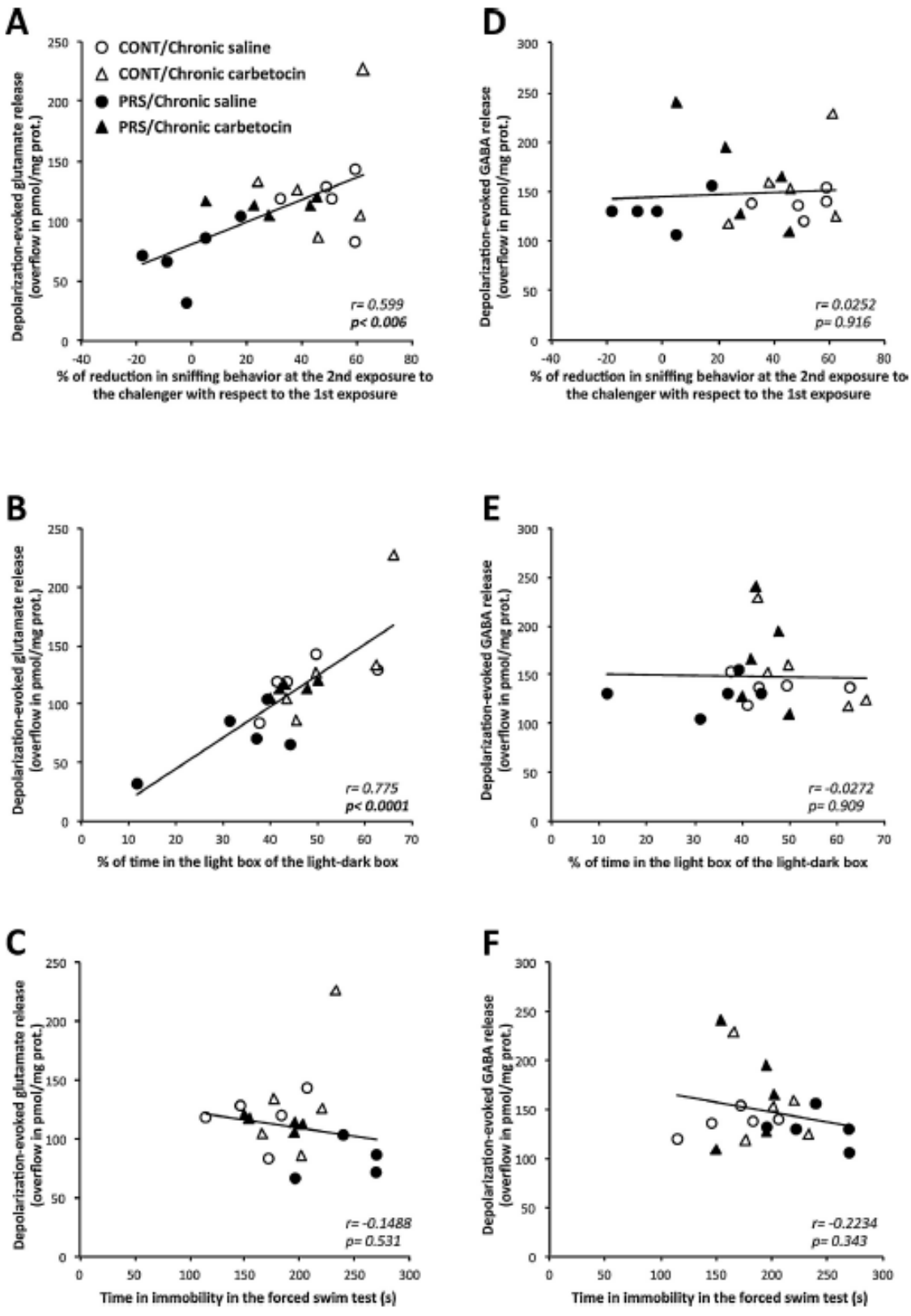

Figure 5: Correlations between glutamate or GABA release and behavioral parameters
Correlations between glutamate or GABA release and social memory (% reduction of sniffing behavior at the second vs. the first exposure to the challenger), anxiety-like behavior, and depressive-like behavior, are shown in (C) and (D), (E) and (F), and (G) and (H), respectively with the r and *p* values of the Pearson's correlation test.

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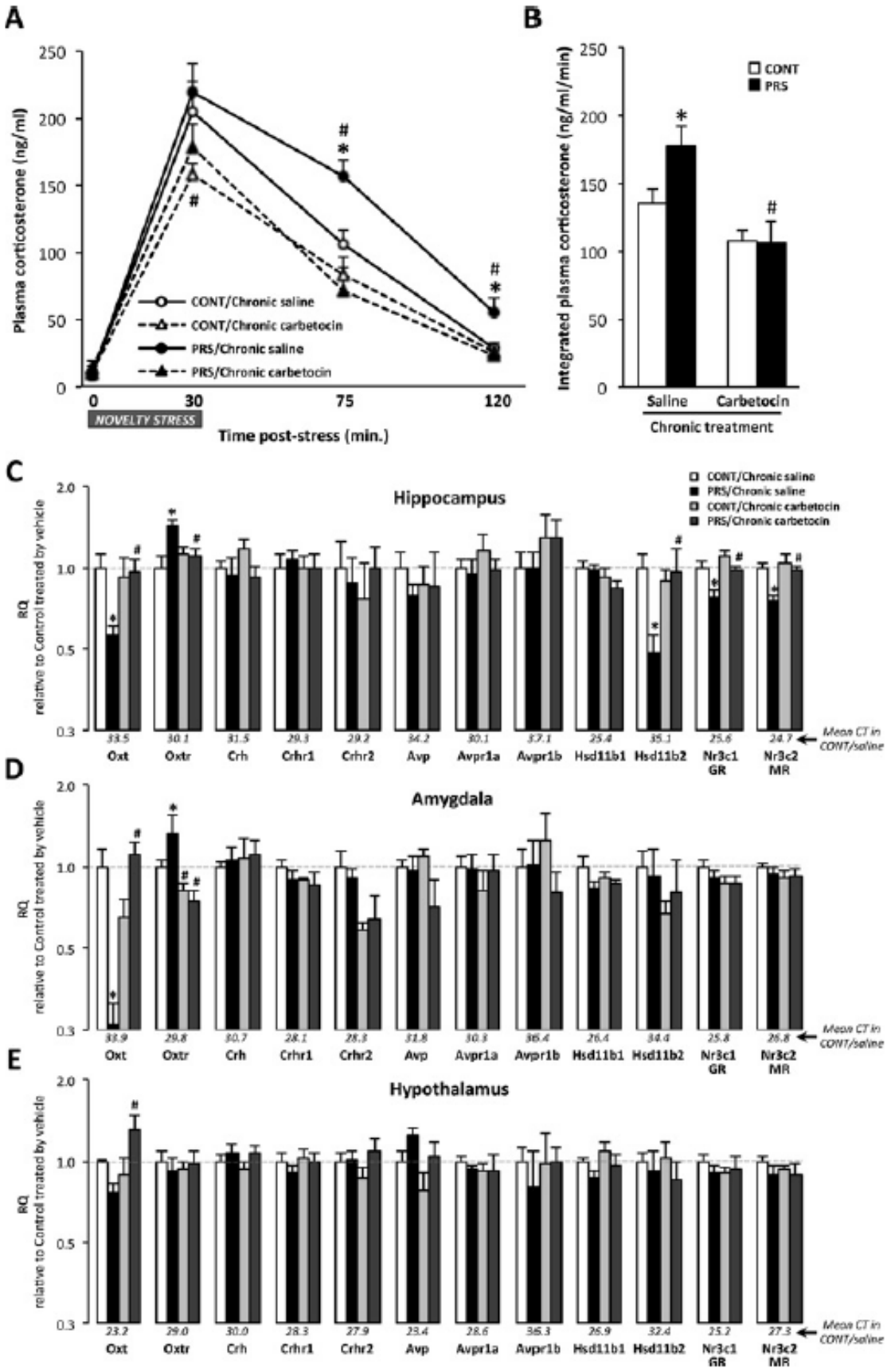

Figure 6: Effect of chronic cabetocin treatment on corticosterone response to novelty stress and associated gene expression analysis in the hippocampus of control and PRS rats.

Plasma corticosterone levels in response to a 30-min exposure to novelty stress (gray box under the timeline) in unstressed control (CONT) and PRS rats treated i.p. with saline or carbetocin (1 mg/kg) daily for 15 days are shown in (A) (group x treatment x time $F_{(3,84)} = 4.17$, $p < 0.05$); Area under the curve (AUC) of integrated plasma levels (see Methods) are shown in (B) (group x treatment x time $F_{(1,28)} = 4.12$, $p < 0.05$). Data are means ± SEM of 8 rats per group. mRNA levels of oxytocin (Oxt), oxytocin receptor (Oxtr), corticotropin releasing hormone (Crh), type-1 and -2 Crh receptors (Crhr1 and Crhr2), vasopressine (Avp), type-1 and -2 Avp receptors (Avpr1a and Avpr1b), type-1 and -2 11β-hydroxysteroid dehydrogenase (Hsd11b1 and Hsd11b2), GR glucocorticoid receptor (Nr3c1), and MR mineralcorticoid receptor (Nr3c2) measured in the hippocampus (C) (Oxt, group x treatment $F_{(1,16)} = 4.2$, $p < 0.05$; Oxtr, group x treatment, $F_{(1,16)} = 6.14$, $p < 0.05$; Hsd11b2, group x treatment, $F_{(1,16)} = 4.72$, $p < 0.05$; Nr3c1, group effect $F_{(1,16)} = 11.63$, $p < 0.01$; treatment effect $F_{(1,16)} = 9.34$, $p < 0.01$; Nr3c2, group effect $F_{(1,16)} = 8.66$, $p < 0.01$; treatment effect $F_{(1,16)} = 6.46$, $p < 0.05$), amygdala (D) (Oxt, group x treatment $F_{(1,16)} = 25.5$, $p < 0.01$; Oxtr, treatment effect, $F_{(1,16)} = 10.98$, $p < 0.01$) and hypothalamus (E) (Oxt, group x treatment $F_{(1,16)}=7.65$, $p<0.01$). Mean cycle threshold (CT) of saline-treated control animals are presented under the histograms. Values are means ± SEM of 5 determinations. Newman keuls post-hoc test: * $p < 0.05$, PRS *vs.* CONT; # $p < 0.05$, Carbetocin *vs.* saline.

GENERAL DISCUSSION

Perinatal environment plays a crucial role in the programming of the offspring. In the present work, we have shown that both pre- and postnatal factors contribute to program the developmental trajectory of the offspring. In particular, high levels of glucocorticoids during fetal life combined to low maternal care in the early postnatal period lead to the development of a pathological phenotype that persists during the whole life of the individual. The model of perinatal stress in rats developed by Prof. Maccari (Maccari et al., 1995) is endowed with face, construct and predictive validity as a model for anxious/depressive disorders, allowing the investigation of the mechanisms that lie at the core of the pathological programming triggered by perinatal stress. Using this model, we were able to demonstrate that activation of OTRs in lactating mothers is able to correct alterations in early maternal care caused by gestational stress, thereby preventing the development of the pathological phenotype across the entire life span. In addition, we could demonstrate that activation of OTRs in the adult offspring reared by stressed dams could correct the anxious-/depressive-like phenotype. We will discuss all these aspects starting from the beneficial effects of carbetocin administration to lactating mothers.

1. The study of carbetocin treatment in lactating mothers discloses a critical role for maternal care in the effect of prenatal stress on the developmental trajectory of the offspring

The strategy of treating lactating mothers with the OTR agonist, carbetocin, moved from the established role of oxytocin in the *peripartum* period. Besides its well-recognized function in promoting lactation, oxytocin behaves as an anti-stress hormone enhancing mother-pup interaction. Hence, we reasoned that pharmacological activation of OTRs in the CNS could improve maternal care particularly in the early lactation period, which critically shapes the neurobiological, endocrinological, and metabolic responses to stress across life. We used carbetocin rather than oxytocin because carbetocin is a highly potent OTR agonist that can cross the blood-brain-barrier (Dvorskà et al., 1992) and shows a longer elimination half-life with respect to oxytocin. The latter feature allows one daily administration of carbetocin. In addition, carbetocin is marketed for the treatment of *postpartum* metrorrhagia (Engstrøm et al., 1998), and is therefore available for human use.

We wish to highlight that the effects of carbetocin treatment to the mother observed in the adult and aged offspring cannot be ascribed to a direct exposure of the pup to the drug because

carbetocin is a synthetic peptide, and the small amount of carbetocin that might be ingested by the pup through the milk is degraded by proteolytic enzymes present in the gut.

In this framework, we could demonstrate for the first time in our model that gestational stress causes a selective impairment in maternal care in the early postnatal period. This impairment was reversed by treatment of the mothers with carbetocin during the first *postpartum* week.

The correction of maternal care by carbetocin was sufficient to prevent all neuroendocrinological and behavioral hallmarks of the pathological phenotype caused by prenatal stress in the offspring. This evidence tips the balance in favor of postnatal events as major determinants of the pathological phenotype induced by prenatal stress. Thus, while the reduced maternal care is a direct consequence of the stress delivered to the mother during gestation, the pathological outcome for the offspring appears to be exclusively mediated by the impairment in mother-pup interaction.

Knowing that the weight at birth influences the developmental trajectory of the individual (Barker et al., 1993; Barker, 2004; Gluckman et al., 2005), one might argue that carbetocin prevents the development of the PRS phenotype simply because it increases the amount of milk delivered to the pups as a result of an overstimulation of OTRs in the mammary gland. However, this was not the case because, unexpectedly, pups fed by mothers treated with carbetocin showed not an increase but a reduction in body weight. Therefore, the beneficial effect of maternal carbetocin was not related to an increased feeding but likely resulted from an improved bonding of developing pups with their mothers. PRS pups fed by carbetocin-treated mothers did not develop any alteration in the resilience to stress across life, as demonstrated by normalization of the HPA response to stress and stress-related behavior.

An important aspect of this study is that carbetocin treatment to lactating mothers could also prevent the long-term outcome of PRS in the aged offspring. We could confirm previous findings showing that aged PRS rats displayed cognitive impairment, as reflected by abnormalities of spatial learning in the water maze and Y maze. This raises the interesting possibility that early-life events may predispose individuals at risk to the development of cognitive decline and dementia during senescence. A growing body of evidence suggests that Alzheimer's disease (AD) and other forms of dementia are associated with a defect in energetic metabolism in brain neurons, and that type-2 diabetes and metabolic syndrome are risk factors for dementia. It has been suggested that glucose uptake in neurons is defective in AD because of an impaired function the type-1 insulin-like growth factor (IGF-1) receptor, which controls membrane translocation of the neuronal glucose transporter GLUT-3 (Giuffrida et al., 2009;

2015). This might result into a reduced energy provision in neurons during synaptic activation as well as into a reduced protein *O*-GlcNAcylation. We therefore examined whether cognitive dysfunctions in our aged PRS rats were associated with metabolic abnormalities and these could be prevented by carbetocin administration to lactating mothers. We found increased glucose plasma levels in PRS rats in response to a mild stressful situation (i.e., immediately after the behavioral performance in the Y maze), which was expected considering the hyperreactivity of the HPA axis displayed by these animals. Glucose levels were normalized in old PRS rats that had been reared by mothers treated with carbetocin. The possibility that glucose uptake consumption in the CNS was altered in PRS rats could not be directly examined by PET imaging with ^{18}F-deoxyglucose at this stage of the research. However, we used an indirect approach by examining protein *O*-GlcNAcylation, a process that is fueled by the glucose entering the cell (Hart et al., 2011; Olivier-Van Stichelen and Hanover, 2015). The ladder of the *O*-GlcNAcylated proteins showed differences between PRS rats reared by mothers treated with saline or carbetocin, which were confined in the molecular range between 170 and 130 kDa. We wondered about the possible significance of this finding and its possible link to cognitive dysfunction of aged PRS rats. To decipher this link we decided to carry out a side project using brain tissue from transgenic mice modeling AD and their wild-type counterparts. We used mice carrying triple mutation of human amyloid precursor protein (APP), presinilin-1, and tau protein (3xTg-AD mice) at an age in which brain pathology is already established and in which a defective brain glucose uptake has been demonstrated (Nicholson et al., 2010). Interestingly, we found remarkable changes in the profile of *O*-GlcNAcylation in hippocampal proteins of 3xTg-AD mice, which was a proof of concept that defective brain glucose consumption within the context of an AD-like phenotype is associated with a decrease in *O*-GlcNAcylation. Because in these mice *O*-GlcNAcylation was also reduced in a molecular weight range containing tau protein, we extended the analysis to the study of the balance between tau protein phosphorylation and *O*-GlcNAcylation (two mutually exclusive posttranslational modification). We found that tau protein was hypo-*O*-GlcNAcylated and hyperphopshorylated in the hippocampus of 3xTg-AD mice. This interesting finding (which is described in Gatta et al., *Pharmacological Research*) strongly encourages the study of tau *O*-GlcNAcylation and phosphorylation in aged PRS rats reared by mothers treated with saline or with carbetocin. This study, which will be a logical follow-up of the present work, may allow establishing whether PRS or early-life stress in general may be considered as a potential risk factor for AD and other types of dementia.

2. Carbetocin: a novel strategy in the treatment of stress-related disorders

I have collaborated to a seminal project in the lab resulting in the reported manuscript published in *The Journal of Neuroscience* (Marrocco et al., 2014). This manuscript demonstrates that both fluoxetine (the prototypical SSRI drug that has been leader in the market of antidepressants for many years) and agomelatine (an atypical antidepressant that acts as a mixed melatonin receptor agonist/5-HT_{2C} receptor antagonist) correct the behavioral phenotype displayed by PRS rats. These findings confer pharmacological validity to the PRS model and make the model suitable for the investigation of novel therapeutic strategies for stress-related disorders, such as OTR activation (see below). The manuscript by Marrocco and colleagues (2014) also shows that antidepressant drugs are able to correct abnormalities in glutamate release and the underlying molecular repertoire in the ventral hippocampus, which a previous manuscript from our group (Marrocco et al., 2012) has indicated as a key neurochemical dysfunction associated with the behavioral outcome of perinatal stress. Taken together, these findings suggest that the regulation of glutamate release in the emotional portion of the hippocampus (the ventral hippocampus) is exquisitely sensitive to long-lasting modifications caused by early-life events, and, more important, that these changes can be reversed by antidepressant medication when the pathological programming is already established and phenotypically apparent, i.e., in the adult life. The finding that glutamatergic transmission is impaired in response to perinatal stress makes also PRS rats a potential model for CNS disorders, such as drug addiction and schizophrenia, in which the neuronal circuit connecting the frontal cortex, the ventral hippocampus, the ventral tegmental area and the ventral striatum are impaired. Research carried out by us and other groups has shown that PRS rats display an impairment in salience attribution and are more vulnerable to drug addiction (Deminière et al., 1992; Morley-Fletcher et al., 2004a; Maccari et al., 2014; Reynaert et al., 2015). PRS rats may therefore be considered as an excellent model for the study of the comorbidity between stress-related disorders and drug addiction. No data are yet available on PRS rats as models of psychosis. However, our group has collaborated with the group of Prof. A. Guidotti at University of Illinois at Chicago in showing that PRS mice recapitulate the epigenetic, neurochemical, and behavioral hallmarks of schizophrenia (Matrisciano et al., 2012).

Data shown in the paper on PRS rats and antidepressant treatment (Marrocco et al., 2014) laid the groundwork for the investigation of the anti-stress and antidepressant-like activity of OTR activation.

A large repertoire of drugs is currently available for the treatment of major depression and, more in general, stress-related disorders. These drugs include SSRIs, serotonin/noradrenaline reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), monoaminooxydase inhibitors, noradrenaline reuptake inhibitors (NARIs), noradrenaline and dopamine reuptake inhibitors (NDRIs), noradrenergic specifically serotonergic antidepressants (NASSAs), atypical antidepressants (agomelatine, trazodone, nefazodone, L-acetyl-carnitine), and, more recently, the dissociative anesthetic, ketamine. In spite of all these drugs, a substantial proportion of depressed patients are refractory to medication with a resulting decrease in the quality of life and a large economic burden for the EU. In addition, drug resistant patients affected by major depression are at risk for suicide.

This encourages the search for novel therapeutic strategies that may target molecular events that lie at the core of the pathophysiology of major depression and correct the abnormal response to stress associated with mood disorders.

Using PRS rats, and moving from the described beneficial effect of carbetocin in the *postpartum* period, we could demonstrate that carbetocin was able to reverse the pathological phenotype caused by early-life stress when systemically administered in the adult life. In our experimental protocol, carbetocin was effective after 3 weeks of treatment similarly to what observed with conventional antidepressant drugs. Further studies are needed to determine the temporal latency of the antidepressant-like effect of carbetocin, a critical issue in the development of novel antidepressant drugs. The possibility that carbetocin has a fast onset of action is suggested by our measurements of glutamate release in isolated synaptosomes prepared from the ventral hippocampus. Contrary to our expectations, application of carbetocin to synaptosomal preparations was able to amplify depolarization-evoked glutamate release, suggesting that OTRs are present not only in GABAergic terminals (Mühlethaler, Charpak and Dreifuss, 1984; Zaninetti and Raggenbass, 2000) but also in glutamatergic terminals. This raises the interesting possibility that pharmacological activation of OTRs directly corrects one of the major neurochemical abnormalities causally related to the pathological phenotype induced by early-life stress, i.e., the reduction of glutamate release in the ventral hippocampus (Marrocco et al., 2012). If so, we predict a rapid onset of action of carbetocin that, if proven, may represent a major breakthrough in the treatment of depression. To the best of our knowledge, only ketamine has shown a fast antidepressant effect in humans (Sanacora and Schatzberg, 2015). However, the use of ketamine in humans is limited by its psychotomimetic effects and its detrimental influence on cognitive functions. OTR agonists lack these adverse

effects and are therefore better candidates for the treatment of patients at high risk of suicide if a fast-onset therapeutic effect is proven.

3. Early-life stress is a triggering factor for (mal)adaptive programming

Present data are in line with the current belief that exposure to adverse events during sensitive developmental time-windows shapes the vulnerability to stress-related disorders and also suggest that early-life stress may be a predisposing factor to cognition disorders later in life. Two hypotheses have been suggested to explain the possible link between early events and late pathologies. The "cumulative stress" or "multiple hit" hypothesis states that aversive experiences early in life predispose individuals to be more vulnerable to additional aversive challenges occurring later in life (McEwen, 1998; Nederhof and Schmidt, 2012). Accordingly, the effects of the environment are cumulative and result into an increased allostatic load, which would enhance the risk for developing a disease. On the other hand, the "match/mismatch" hypothesis states that stressful experiences early in life trigger adaptive processes, thereby allowing the individual to survive to aversive challenges later in life (Belsky and Pluess, 2009; Schmidt, 2011). A reduced resilience to acute stress may be the price to pay in this process. From an evolutionary perspective, the hallmarks of major depression, i.e., psychomotor retardation, reduced motivation, alterations in sleep and feeding behavior, and cognitive dysfunction, may represent the "key to survival" for "loser" individuals in a hierarchic struggle for resources (reviewed by Krishnan and Nestler, 2010). Exposure to low maternal care or other early adverse events might predispose an individual to be a "loser", but, on the other hand, might set the epigenetic program for survival, which is phenotypically expressed as a set of behavioral paradigms that are hallmarks of anxious/depressive disorders. According to this view, the programming triggered by early-life stress cannot be considered as "maladaptive" in groups of animal in which hierarchy is exclusively established on the basis of the physical resources (i.e., the "dominant" is the strongest animal). Humans, however, live in a different social context where hierarchy may not rely on physical resources, and, therefore, the anxious/depressive phenotype developing in response to early-life stress may lose the original evolutionary significance becoming detrimental for self-care and rewarding social interactions. Ironically, this program that in humans may be no longer linked to a defensive strategy may preserve the main feature of evolution, which is the transmission to the progeny. In other words, an individual may show an anxious/depressive phenotype without any apparent exposure to early-life stress because of the transgenerational transmission of the epigenetic programing. The "optimal" antidepressant strategy should hit the core of the epigenetic

programming triggered by early-life stress either at the onset of the programming or when the expression of the programming has been established. Epigenetic drugs, i.e., drugs that interfere with mechanisms of DNA methylation or histone acetylation are obvious candidates, but, at the present stage they lack any specificity unless they are locally injected into target brain regions. Of note, however, the acetylating agent, L-acetylcarnitine, was shown to cause a fast-onset antidepressant-like effect in both rats and mice (Cuccurazzu et al., 2013; Nasca et al., 2013). The oxytocinergic system may represent an alternative valuable target for drugs aimed at "fighting the noumenon of stress-related disorders" by providing a link between early life events (particularly related to maternal care) and the "pathological" phenotype of the adult life. Remarkably, we have found that adult PRS rats show a reduction in the transcript encoding for oxytocin and a compensatory increase in the transcript encoding for OTRs in the hippocampus, suggesting that the oxytocinergic system is amenable to treatment with oxytocin itself or with synthetic drugs that potently and selectively activate OTRs. A clear advantage of targeting the oxytocinergic system is that oxytocin behaves as an anti-stress hormone and enhances interindividual bonding thereby improving rewarding social interactions. Oxytocin and OTR agonists might be administered to the mother in the early *postpartum* period if needed, or, alternatively, in the adult life to patients affected by anxious/depressive disorders. We predict that treatment with oxytocin or OTR agonists may cause a fast-onset antidepressant effect being effective in a larger percentage of patients as compared to currently marketed antidepressants. This, however, remains to be investigated. Last, but not least, oxytocin or OTR agonists are expected to exhibit a favourable profile of safety and tolerability lacking all major adverse effects typically induced by antidepressant drugs, such as body weight gain, sexual dysfunction, and cardiovascular adverse effects *inter alia*.

Intranasal oxytocin is currently used in humans in the experimental treatment of CNS disorders including autism spectrum disorders and schizophrenia (reviewed by Matsusaki et al., 2012; De Berardis et al., 2013; Lee et al., 2015). Our findings strongly encourage the experimental use of oxytocin or OTR agonists in the treatment of anxious/depressive disorders in humans.

Perspectives

In summary, the following findings were generated by this PhD project: (*i*) pharmacological activation of oxyocin receptors in lactating mothers could correct the defect maternal care caused by gestational stress; (*ii*) this treatment could also prevent the pathological phenotype caused by prenatal stress across the entire life span of the offspring; (*iii*) this also includes cognitive dysfunctions, abnormalities in glucose metabolism, and a reduced protein *O*-GlcNAcylation showed by the aged offspring reared by mothers subject to gestational stress; and (*iv*) carbetocin administration in the adult life could also correct the pathological phenotype caused by prenatal stress and the underlying reduction in glutamate release in the ventral hippocampus, thus mimicking the effect of conventional antidepressant medication. As a by-product of the project, we could also demonstrate a reduced *O*-GlcNAcylation of hippocampal proteins, including tau protein, in a genetic mouse model of Alzjeimer's disease. These data lay the groundwork for additional studies that we believe are necessary to support the value of targeting oxytocin receptors in the treatement of psychiatric disorders.

First, it will be necessary to determine whether the oxytocinergic system in the hippocampus and other brain regions becomes defective in lactating mothers exposed to gestational stress. Second, it will be important to determine when precisely the defect of hippocampal oxytocinergic system develops during maturationof the offspring reared by mothers exposed to gestational stress. Third, it will be essential to unravel the molecular nature of the epigenetic programming triggered by perinatal stress in the offspring and how correction of maternal care or pharmacological activation of oxytocin receptors later in life impacts on these molecular determinants. Fourth, the "therapeutic" action of carbetocin or oxytocin should also be examined in other rats models of mood disorders such as spontaneously depressed Flinders Sensitive Line rats (Overstreet and Wegener, 2013). Fifth, from a therapeutic perspective, it is particularly important to perfrom time-dependent studies evaluating the effect of carbetocin treatment in the adult life. As we have highlighted before, a major limitation associated with the use of most of the current antidepressants, is that at least three weeks are necessary to improve mood in depressed patients. We predict, that pharmacological activation of oxytocin rceeptors may produce a faster therapeutic effect, but this remains to be demonstrated. Sixth, experiments in which carbetocin or oxytocin are locally injected in different brain regions of PRS rats are necessary to support our hypothesis that the hippocampus is central to the action of this drug. Seventh, it will be important to determine the identity of hippocampal proteins that are hypo-*O*-GlcNAcylated in response to perinatal stress. Eighth, measurments of 2-

deoxyglucose uptake should be performed by PET or autoradiography to examine whether perinatal stress causes alterations in brain glucose consumption in aged rats. Nineth, the study should be extended to mice exposed to gestational stress, which will facilitate the examination of the effects of carbetocinor oxytocin in genetic models of human disorders in the elderly such as Alzheimer's disease and frontotemporal dementia.

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Abstract

Both prenatal and postnatal factors (e.g., the transplacental transfer of molecules to the fetus and the extent and quality of matenal care, rexpectively) contribute to program the developmental trajectory of the offspring. This trajectory extends to the old age, which is the age at maximal risk for the clinical onset of chronic neurodegenerative disorders, such as Alzheimer's disease (AD).

A growing body of evidence has revealed the role of oxytocin as an antistress factor. In addition, during the *postpartum* period, maternal oxytocin plays a key role in mother-pup interactions that highly contribute to the development of the brain and shape the response of the hypothalamic-pituitary-adrenal axis to stress in the offspring. Using the model of prenatal stress in rats (PRS), we showed that postnatal administration of the oxytocin receptor agonist, carbetocin, to stressed mothers improved maternal behavior and prevented the pathological consequences of early-life stress across the entir lifespan of the offspring. These consequences include an anxious-/depressive-like phenotype as well as cognitive impairments and metabolic abnormalities in the aged offspring. We also demonstrated that chronic carbetocin treatment in adult rats was also able to correct the behavioral consequences of PRS and the underlying reduction in glutamate release in the ventral hippocampus, thus mimicking the action of the antidepressants fluoxetine and agomelatine.

Because we found a reduction in protein *O*-GlcNacylation in the hippocampus of aged PRS rats showing cognitive dysfunction, we also decided to examine whether a similar phenomenon was present in animals modeling AD. As a by-product of the main project we were able to demonstrate that AD mice carrying a triple mutation of amyloid precursor protein, presenilin-1 and tau show a selective reduction of protein *O*-GlcNacylation in the hippocampus. In particular, tau protein was hypo-*O*-GlcNacylated and hyperphosphorylated in the hippocampus of these mice.

In conclusion, our data demonstrate that perinatal stress may represent a risk factor for psychiatric and neurodegenrative disorders and that carbetocin adminitration to either lactating mothers or the adult offspring may eliminate this risk. This raises the attractive possibility that mothers exposed to stress during gestation or in the early *postpartum* period or developing disorders that reduce maternal care such as *postpartum* depression should be treated with oxytocin receptor agonists to prevent the pathological consequences of a defective maternal care for the developing child.

Résumé

Les facteurs pré- et postnataux (tels que le passage de molécules à travers la barrière placentaire ainsi que le degré et la qualité des soins maternels) contribuent tous deux à programmer le développement de la descendance. Cette programmation perdure jusqu'au vieillissement, période de risque maximal pour l'apparition clinique de maladies neurodégénératives chroniques telles que la maladie d'Alzheimer (MA).

De nombreuses études ont mis en évidence les propriétés antistress de l'ocytocine. En particulier, pendant la période périnatale, l'ocytocine maternelle intervient dans la mise en place des interactions mère-petit. Le comportement maternel joue un rôle important dans le développement du cerveau de la descendance et participe à la mise en place de la réponse de l'axe hypothalamo-hypophyso-surrénalien au stress. En utilisant le modèle de stress périnatal chez le rat (PRS), nous avons montré que l'administration postnatale à des mères stressées d'un agoniste du récepteur à l'ocytocine (la carbétocine) améliore le comportement maternel et empêche les conséquences néfastes du stress précoce tout au long de la vie de la descendance. Cela comprend le phénotype de type anxieux/dépressif, le déclin cognitif ainsi que les troubles métaboliques de la descendance âgée. Nous avons également démontré qu'un traitement chronique à la carbétocine durant la vie adulte corrige les altérations comportementales et la réduction de la libération de glutamate dans l'hippocampe ventral des rats PRS. Ainsi, la carbétocine a un effet similaire à celui des antidépresseurs conventionnels (fluoxétine, agomélatine). Au vu de la réduction en protéines *O*-GlcNacylées observée dans l'hippocampe des rats PRS âgés (présentant également un déclin cognitif), nous nous sommes alors demandés si un mécanisme similaire pouvait se retrouver dans des modèles animaux de la MA. Ainsi, nous avons pu démontrer que les souris portant une triple mutation sur la préséniline-1, la protéine précurseur de l'amyloïde, et la protéine tau montrent une réduction sélective de la *O*-GlcNacylation des protéines hippocampiques. En particulier, la protéine tau est hypo-*O*-GlcNAcylée et hyperphosphorylée dans l'hippocampe des ces souris.

En conclusion, l'ensemble de nos résultats montre que le stress périnatal peut représenter un facteur de risque pour le développement de troubles psychiatriques et des atteintes neurodégénératives. De manière intéressante, le traitement à la carbétocine chez des mères allaitantes, ou chez leur descendance adulte peut éliminer ce risque. Cela suggère que les mères exposées à un stress soit pendant la gestation, soit pendant la période postnatale, ou encore atteintes de troubles qui réduisent les soins maternels tels que la dépression *postpartum* pourraient être traitées par des agonistes des récepteurs à l'ocytocine afin de prévenir les conséquences néfastes induites par un soin maternel défectueux sur le développement de l'enfant.