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Scuola di dottorato farmacologia e tossicologia

Double-PhD Thesis

Defended on September 28th, 2020 by

Rémy VERHAEGHE

ROLE OF EARLY-LIFE STRESS AND METABOTROPIC GLUTAMATE RECEPTORS IN THE DEVELOPMENTAL TRAJECTORY OF THE CENTRAL NERVOUS SYSTEM

Members of the Jury:

Prof. Paolo CALABRESI, MD	Policlinico Universitario Agostino Gemelli IRCCS Italy	***President of the Jury***
Prof. Anna PITTALUGA, PhD	University of Genoa, Italy	***Reporter***
Prof. Richard N'GOMBA, PhD	University of Lincoln, United Kingdom	***Reporter***
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ABSTRACT

Stressful events occurring during perinatal life programs the emergence of pathological phenotypes later in life. By using the perinatal stress rat model, we investigated the long-lasting effects of perinatal stress (PRS) on the glutamatergic synapse in males and females. Remarkably, we demonstrated that long-term programming of PRS is strictly sex-dependent and induce a dysmasculinization of the glutamatergic synapse whereas old females PRS rats were protected. Because motor functions decrease during aging, we further investigated the long-term effects of PRS on the basal ganglia, a group of subcortical nuclei involved in motor functions. We could demonstrate that perinatal programs an accelerated aging of the basal ganglia motor system. Since metabotropic glutamate receptors 2/3 (mGlu2/3) are constantly decreased by PRS across lifespan in both sexes, we studied the role of mGlu2 and mGlu3 in brain development. We showed that mGlu3 receptors shape the developmental trajectory of cortical GABAergic system. Considering our findings showing that mGlu3 receptors and early life stress influence the brain development, we investigated how the interplay between these environmental and genetic factors impact neuroplasticity markers during development in hippocampus, a central region in stress response. We observed that mGlu3 and maternal restraint stress (MRS) alter GABAergic interneurons related genes expression, stress and epigenetic markers. Surprisingly, mice lacking mGlu3 receptors submitted to MRS showed compensatory mechanisms during specific time window during development. Taken together our results strengthen the idea that nature and nurture shape brain development.

Université de Lille : Sciences et Technologies
École Doctorale BIOLOGIE SANTÉ (ED n° 446)

UNIVERSITÉ DE LA SAPIENZA
Ecole doctorale de pharmacologie et toxicologie

PRENATAL STRESS AND NEURODEGENERATIVE DISEASES
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Rémy VERHAEGHE

RÔLE DU STRESS PRÉCOCE ET DES RÉCEPTEURS MÉTABOTROPIQUES AU GLUTAMATE DANS LA TRAJECTOIRE DÉVELOPPEMENTALE DU SYSTÈME NERVEUX CENTRAL

Membres du Jury :

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RESUME

Les événements stressants survenant au cours de la vie périnatale programment l'émergence de maladies au cours de la vie. En utilisant le modèle de rat de stress périnatal, nous avons étudié les effets à long terme du stress périnatal (PRS) sur la synapse glutamatergique chez les mâles et les femelles. Remarquablement, nous avons démontré que la programmation à long terme du PRS est strictement dépendante du sexe et induit une démasculinisation de la synapse glutamatergique alors que les femelles PRS âgés étaient protégées. Parce que les fonctions motrices diminuent au cours du vieillissement, nous avons approfondi les effets à long terme du PRS sur les ganglions de la base, un groupe de noyaux sous-corticaux impliqués dans les fonctions motrices. Ainsi, nous avons pu démontrer qu'un stress périnatal induit un vieillissement accéléré du système moteur des ganglions de la base. Étant donné que les récepteurs métabotropiques au glutamate 2/3 (mGlu2/3) sont constamment diminués par le PRS chez les mâles et femelles au cours de la vie, nous avons étudié le rôle des récepteurs mGlu2 et mGlu3 dans le développement du cerveau. Nous avons montré que les récepteurs mGlu3 façonnent la trajectoire développementale du système GABAergique cortical. Compte tenu de nos résultats montrant que les récepteurs mGlu3 et le stress précoce influencent le développement du cerveau, nous avons étudié l'influence de l'interaction entre ces facteurs environnementaux et génétiques sur les marqueurs de la neuroplasticité lors du développement dans l'hippocampe, une région centrale de la réponse au stress. Nous avons observé que les récepteurs mGlu3 et le stress précoce (MRS) altèrent l'expression des gènes liés aux interneurones GABAergiques ainsi que les marqueurs du stress et les marqueurs épigénétiques. Étonnamment, les souris dépourvues de récepteurs mGlu3 soumis au MRS ont montré des mécanismes compensatoires pendant une fenêtre de temps spécifique au cours du développement. Ensemble, nos résultats renforcent l'idée que les gènes et l'environnement façonnent le développement du cerveau.

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Education

2016 -2020 **PhD student in Neuroscience and Pharmacology**, double-PhD thesis between Sapienza University of Rome (Italy) and Lille University (France)

2015 **Master's in Biology and Health**, specialty in Physiology and Physiopathology of Human Diseases, Lille University

2013 **Bachelor's Degree in Biology,** Lille University. Research label, obtained for participating at several research conferences

2011 **Technological University Degree (DUT) in Chemistry** (Bethune, France)

2009 **Baccalauréat of Sciences**, Châtelet High School (Douai, France)

Work Experience

2015-2016 **Research Engineer**, Neuromed Institute (Italy)

2016-2020 **Double-PhD thesis student** at Lille University and Sapienza University of Rome. Thesis: Early life stress and metabotropic glutamate receptors in the developmental trajectory of the central nervous system, under the co-supervision of Profs. Ferdinando Nicoletti and Stefania Maccari. Funded by Neuromed (IRCCS NEUROMED Mediterranean Neurological Institute).

Projects at the Neuromed Institute and Sapienza University: 1) Study of the role of metabotropic glutamate receptors (mGlu receptors) in the cortical GABAergic interneurons development. 2) Investigating the interplay between maternal restraint stress and genetic deletion of mGlu3 receptors on the developmental trajectory of the GABAergic system in hippocampus.

Projects at Lille University: 1) Study the long-lasting effects of perinatal stress on the glutamatergic synapse in prefrontal cortex and hippocampus in aged male and female rats. 2) Investigating long-term effects of perinatal stress on the basal ganglia motor circuit in adult and aged rats.

2015 **Research Intern,** six months during my master 2 recherche, at the "Glycobiology of Stress-related Disorders" team (Prof. Stefania Maccari, Lille Univ.). Project: study the long-lasting behavioral effects of perinatal stress (PRS) in aged male and female rats, and the involvement of the glutamatergic and dopaminergic synapse in the hippocampus, prefrontal cortex and the striatum.

2014 **Research Intern,** six weeks during my master 1st year, at the "Glycobiolgy of Stress-related Disorders" team. Project: Investigated perinatal stress effects in rats and glutamatergic hypothesis in stress related alterations induced by PRS (supervisor: Prof Stefania Maccari).

2013 **Research Intern,** six weeks, during my bachelor 3rd year in the laboratory of Functional Neurosciences and Pathologies (supervisor: Prof. Muriel Boucart, Lille Univ.). Project: studied peripheral vision in healthy individuals by oculometry.

2013 **Research Intern,** one month during my bachelor 3rd year. Alzheimer & Tauopathies team (Prof. Luc Buee, Lille Univ.) Project: investigation of epigenetic marks in an animal model of Alzheimer's disease. (supervisor: Dr Marie Christine Galas)

Publications

Genetic deletion of mGlu2 metabotropic glutamate receptors improves the short-term outcome of cerebral transient focal ischemia. Mastroiacovo F, Moyanova S, Cannella M, Gaglione A, Verhaeghe R, Bozza G, Madonna M, Motolese M, Traficante A, Riozzi B, Bruno V, Battaglia G, Lodge D, Nicoletti F. *Mol Brain*. 2017 IF : 4.1

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Interplay between mGlu3 receptor and maternal restraint stress on GABAergic system development in mice hippocampus. Verhaeghe R, Imbriglio T, Maccari S, Battaglia G, Cannella M, Nicoletti F. *In preparation.*

Talks

2018 **Programming of the glutamatergic, GABAergic and dopaminergic synapse along life-span: genetic vs. environmental approaches**. 1st workshop "When neuroscience meets Glycobiology" at the University of Lille.

2018 **Genetic and environmental factors shape the brain development**. 21th EURON PhD Days at the Catholic University of Louvain in Belgium

2019 **Perinatal stress programs sex differences in the glutamatergic synapse and related behaviors during aging**. 22th EURON PhD Days at the Luxembourg University

Posters

2019 Society for Neuroscience meeting, San Diego

Perinatal stress programs sex differences in the glutamatergic synapse pf brain regions and related behaviors during aging. Remy Verhaeghe, Sara Morley-Fletcher, Hammou Bouwalerh, Gilles Van Camp, Charlotte Clarisse, Francesca Cisani, Vance Gao, Yann Guerardel, #Ferdinando Nicoletti, Stefania Maccari

Early life stress causes a long-lasting dopaminergic synaptopathy in the striatal motor circuit and related behaviours Stefania Maccari, Sara Morley-Fletcher, Jordan Marrocco, Remy Verhaeghe, Gilles Van Camp, Hammou Bouwalerh, Domenico Bucci, Milena Cannella, Anna Pittaluga, Giuseppe Battaglia, Ferdinando Nicoletti

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2019 PhD Days, Lille

Perinatal stress programs sex differences in the glutamatergic synapse of brain regions and related behaviors during aging. Remy Verhaeghe, Sara Morley-Fletcher, Hammou Bouwalerh, Gilles Van Camp, Charlotte Clarisse, Francesca Cisani, Vance Gao, Yann Guerardel, #Ferdinando Nicoletti, Stefania Maccari

2018 Society for Neuroscience meeting, San Diego

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2017 Society for Neuroscience meeting, Washington

Expression of GABAergic interneuron-related genes in mice with genetic deletion of mGlu2 and mGlu3 metabotropic glutamate receptors Cannella M, Imbriglio T, Verhaeghe R, Bucci D, Scalabri F, Simeone A, Maccari S, Battaglia G, Nicoletti F

2017 International Meeting on Metabotropic Glutamate Receptors, Taormina, Italy

Expression of GABAergic interneuron-related genes in mice with genetic deletion of mGlu2 or mGlu3 metabotropic glutamate receptors M. Cannella, T. Imbriglio, R. Verhaeghe, M. Quattromani, D. Bucci, F. Scalabrì, A. Simeone, S. Maccari, T. Wieloch, G. Battaglia, F. Nicoletti

NeuroFrance 17-19 Mai 2017 Bordeaux

Perinatal stress causes cognitive dysfunctions during ageing in rats reversed by oxytocin agonist. Van Camp G, Bouwalerh H, Morley-Fletcher S, Gatta E, Verhaeghe R, Mairesse J, Nicoletti F, Maccari S

Lifelong effect of perinatal stress on basal ganglia motor function in rats Morley-Fletcher S, Bouwalerh H, Van Camp G, Marrocco J, Cannella M, Motolese M, Battaglia G, Ravasi L, Verhaeghe R, Nicoletti F, Maccari S

2016 International Symposium on Research on Healthy Ageing, Lille

Lifelong effect of perinatal stress on basal ganglia motor function in rats Maccari S, Marrocco J, Verhaeghe R, Cannella M, Motolese M, Battaglia G, Ravasi L, Bouwalerh H, Van Camp G, Morley- Fletcher S, Nicoletti F

Languages and Hobbies

French: Native
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Italian: C1 (Able to communicate fluently and without difficulty)
Hobbies: Tennis (club and competition), nature travel, cinema

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GENERAL SUMMARY

Brain development and behavioral responses are shaped by a complex interaction between genes and environment. Stressful events occurring during prenatal and early post-natal life (e.g. perinatal period) contribute to program the developmental trajectory of the offspring. Adverse environment during perinatal period can lead to maladaptive responses resulting in an increase of the risk to develop diseases such as metabolic diseases (*Barker et al., 1995*) or psychiatric disorders involving the postnatal human relationship (*Maccari et al., 2017*).

In order to investigate programming effects triggered by early exposure to environmental stressors, animal models of early life stress have been designed. In this project, we used the perinatal stress model in rats, described by Prof Stefania Maccari (*Maccari et al., 1995*). Perinatal stress paradigm in rats consists of submitting pregnant dams to multiple episodes of restraint stress under bright light. 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.

PRS induces glutamatergic synaptic transmission abnormalities in ventral hippocampus, a brain region encoding memories related to stress and emotions (*Fanselow and Dong, 2010*), thereby enhancing the vulnerability to stress-related disorders in adult life (*Marrocco et al., 2012*; *Marrocco et al., 2014*; *Mairesse et al., 2015*; *Morley-Fletcher et al., 2018*). PRS triggers a decrease of glutamatergic release in ventral hippocampus associated with a reduction of risk-taking behavior (*Marrocco et al., 2012*). Remarkably, alterations of risk-taking behavior were abolished by reversing glutamatergic release in ventral hippocampus (*Marrocco et al., 2012*) suggesting that glutamatergic synapse impairment lies at the core of the altered phenotype caused by early life stress. Consistently with the idea that glutamatergic system plays a central role in PRS, metabotropic glutamate receptors 2/3 (mGlu2/3) are reduced by PRS in pups (*Laloux et al., 2012*) and in adult males and females (*Zuena et al., 2008*). Programming effects induced by PRS diverge between males and females. For example, at a behavioral level, PRS decrease risk-taking behavior in males whereas it increases risk-taking behavior in females. Sex differences are also observed in addictive like behavior (*Reynaert et al., 2016*). Besides these behavioral changes, other 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*) suggesting that PRS could affect aging related processes. Alteration of the nigrostriatal system represents a risk factor for age-related disorders. Several evidences suggest that early life stress modulate the nigrostriatal system. Indeed, PRS affects the symmetry of D_2 receptor expression in the medial caudate/putamen (*Adrover et al., 2007*), and to restrain haloperidol-induced catalepsy and enhance apomorphine-induced stereotypies (*Marrocco et al., 2013*) in adult rats.

Additionally, to environmental factors, genes shape brain development. Correct balance between neuronal excitation and inhibition is critical for neuronal networks formation. Because mGlu2 and mGlu3 receptors regulate glutamate release, these two receptors are very important in the excitation/inhibition (E/I) balance. Neuronal inhibition mainly relies on GABAergic neurotransmission. While GABA is essentially known for its inhibitory effects, it acts as an excitatory neurotransmitter during development. Hence, GABA is essential for sculpting neuronal connections during brain development. Although glutamate and GABA systems influence together E/I balance, the role of mGlu3 and mGlu2 receptors on GABAergic system development is poorly understood.

Within this context, the main objective of my PhD program was to establish the role of early life stress and metabotropic glutamate receptors on the central nervous system development across life span.

In the **chapter 1** of the thesis, we could demonstrate that lifelong programming triggered by early life stress is sex-dependent in aged rats. This is remarkably reflected by the dysmasculinization of the glutamatergic synapse induced by PRS in males whereas females PRS displayed protective effects during aging. Since motor functions degrade during aging, we further investigated the effects of PRS on basal ganglia motor system and motor behaviors. We showed that PRS programs an accelerated aging of the basal ganglia motor system.

These findings are particularly important because of the progressively increased of the aged population. Understanding how early life environment make individuals more vulnerable to age-related disorder could be very helpful for the quality of life of our modern society. Our data may provide a first step into this direction.

Because metabotropic glutamate receptors 2/3 (mGlu2/3) are constantly decreased by PRS across lifespan in both sexes, we further investigated the role of mGlu2 and mGlu3 in brain development in **chapter 2**. We could demonstrate that mGlu3 receptors shape the

developmental trajectory of cortical GABAergic system. These results could encourage a pharmacological approach targeting mGlu3 receptors in order to correct developmental abnormalities of GABAergic system observed in neuropsychiatric disorders such as schizophrenia.

The last part of the project, in **chapter 3**, was to investigate the influence of the interplay between maternal restraint stress (MRS) and mGlu3 receptors on neuroplasticity genes expression in hippocampus, a central brain region involved in stress response. We showed that mGlu3 and MRS alter GABAergic interneurons related genes expression, stress and epigenetic markers. Interestingly, mice lacking mGlu3 receptors submitted to MRS showed compensatory mechanisms during specific time window during development.

Taken together, our results indicate that 1) long-lasting programming effects induced by PRS are sex-dependent, 2) PRS accelerates aging of the basal ganglia motor system, 3) mGlu3 receptors shape the developmental trajectory of cortical GABAergic system, and, 4) MRS and mGlu3 influence together the expression of neuroplasticity genes in hippocampus during development.

Hence, our results may help to understanding sex-differences and to introducing new therapeutic and research approaches also in clinic aiming to reverse central nervous system developmental abnormalities and to reduce vulnerability to aged related disorders. Finally, understanding how perturbations of the early life environment, shape the vulnerability to age-related disorders could be very helpful for the quality of life of our modern society.

GENERAL INTRODUCTION

I. STRESS

1. STRESS INTRODUCTION

We live in a constantly changing environment. In order to adapt, the organism evaluate these environmental modifications as a threat or not and react in consequence. In 1915, Walter Bradford Cannon coined the term fight or flight to describe an animal's response to threats. In "Bodily Changes in Pain, Hunger, Fear and Rage" he explained that the fight or flight response is a physiological reaction that occurs in response to a perceived harmful event, attack, or threat to survival. When animals perceive an event as harmful, as an attack or as a threat, a physiological reaction occurs characterizing by a general discharge of the sympathetic nervous system, priming the animal for fighting or fleeing in order to survive (*Canon, 1915*).

In 1926, Walter Bradford Cannon extended Bernard's "milieu intérieur" concept to *homeostasis* (*homeo* meaning "similar" and *stasis* for "standing still"). He wrote "*The word does not imply something set and immobile, a stagnation. It means a condition - a condition which may vary, but which is relatively constant"* (*Canon 1926*; *Canon 1932*). Some years later, in 1936, Hans Selye, the "father of stress", described "a syndrome caused by nocuous agent" that was the premise of what will be called later the stress response (*Selye 1936*). Using acute non-specific nocuous agents in rats Hans Selye observed a typical syndrome that can be divided in three stages. The General Adaptation Syndrome (GAS) is a profile of how organisms respond to stress; GAS is characterized by three phases:

1) a nonspecific mobilization phase, which promotes sympathetic nervous system activity
2) a resistance phase, during which the organism makes efforts to cope with the threat
3) an exhaustion phase, which occurs if the organism fails to overcome the threat and depletes its physiological resources. (*Selye 1950*)

Because the concept of homeostasis didn't fit with the long-term effects of semi-chronic or chronic stress, an effort from the scientific community has been made later to established new concepts that explain the physiological changes induced by stress.

2. ALLOSTASIS AND ALLOSTATIC LOAD

When a chronic stress occurs, physiological parameters are modified and tend to reach a new stability when stress ends. For example, during stress the blood pressure increases. If stress becomes chronic it leads to the adaptation of the organism. In this purpose the blood pressure baseline reachs a new equilibrium and is higher than previously as seen in rats (*Folkow and Rubinstein, 1966*) and in monkeys (*Hoffbrand and Forsyth 1969*). Hence, the organism will be prepared to respond with some form of coping behavior to an environmental challenge that generally requires a rise in blood pressure. So rather than maintaining constant physiological parameters (homeostasis), the organism must vary all the parameters of its internal milieu and match them appropriately to environmental demands. This concept has been called allostasis (allos for "other" and stasis for "standing still"), meaning "stability through change" (*Sterling and Eyer, 1988*).

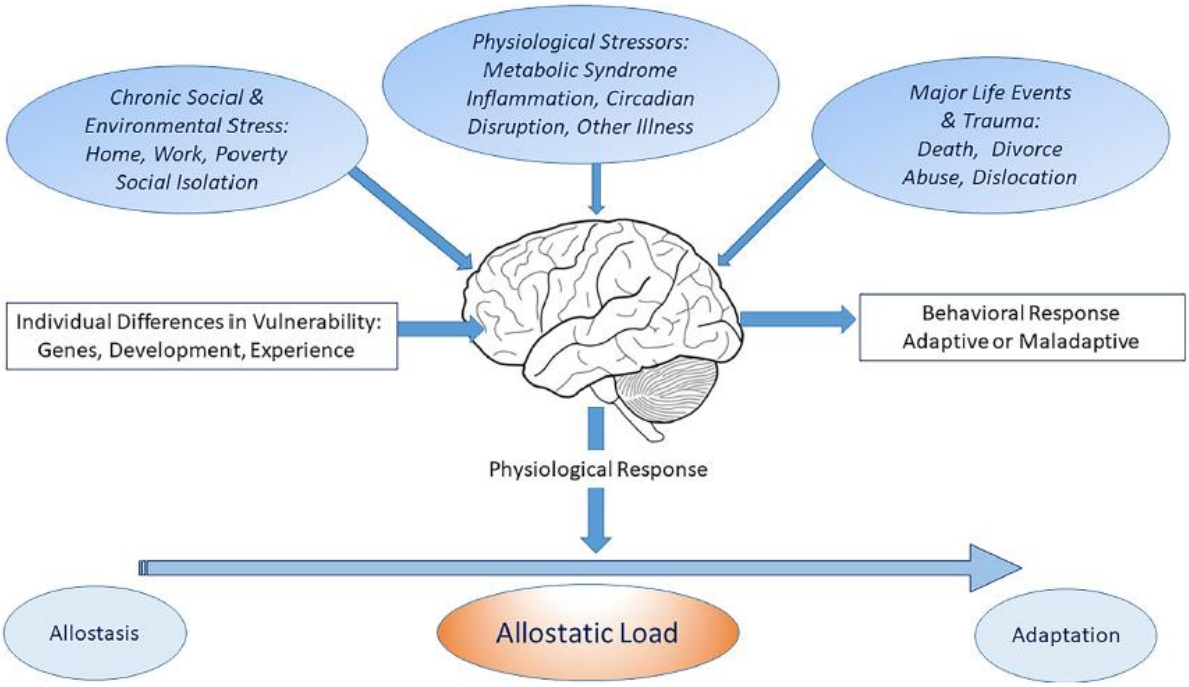

Figure 1. The brain is a primary organ that perceives and responds to what is stressful to an individual. The major function of cortisol and other mediators of allostasis is to promote adaptation. However, overuse and/or dysregulation among the mediators of allostasis lead to allostatic load (or overload) and accelerate disease processes such as cardiovascular disease, diabetes, and affective disorders. Three limbic brain regions are noted. (*From McEwen & Akil, 2020*)

Reaching this new equilibrium is costly to the organism. Responding to stress costs energy from the organism and if this process becomes chronic, it predisposes individuals to chronic

diseases. Bruce Mc Ewen reinforced the concept of allostasis describing the allostatic load. He stipulated that "allostatic load refers to the price the body pays for being forced to adapt to adverse psychosocial or physical situations" (*McEwen, 2000*). As depicted in figure 1, individuals along their life face many types of stress, such as chronic social, physiological stress and even trauma. Each individual responds to stress differently depending on genes, development and experience. They use different coping strategies determined by their individual differences in vulnerability and display an adaptive or maladaptive behavioral response. The more that individuals face stress during their lives the more the allostatic load is elevated, and thus, individuals will be more prone to develop chronic diseases.

3. STRESS CIRCUITRY

Over time, the human stress response has evolved to maintain homeostasis under conditions of real or perceived stress. This objective is achieved through autoregulatory neural and hormonal systems. The hypothalamic-pituitary-adrenal (HPA) axis is a key regulatory pathway in the maintenance of these homeostatic processes.

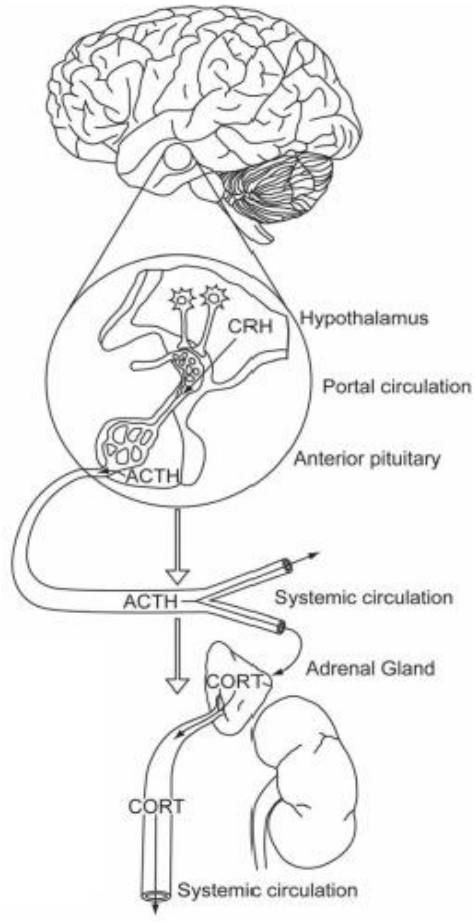

Figure 2. Organization of the hypothalamo-pituitary-adrenocortical (HPA) axis (*from Herman et al., 2016).*

The HPA axis is activated by the stimulation of neurons in the medial parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus. Neurons in the PVN secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the portal vessel system. These neurohormones trigger the synthesis of pro-opiomelanocortin (POMC) in the anterior pituitary, which is cleaved to corticotropin (ACTH), opioid and melanocortin peptides, including other peptide hormones. ACTH initiated the release of glucocorticoids from the adrenal cortex and adrenaline and noradrenaline from the adrenal medulla. When stress ends, the HPA axis returns to a baseline state by the action of several feedback loops. In order to shut down the

activation of HPA axis glucocorticoids bind to glucocorticoids receptors in hippocampus and frontal cortex that project back to the hypothalamus. Adrenal glucocorticoid hormones are crucial for maintenance of homeostasis and adaptation to stress. Glucocorticoids act through two receptors subtypes; the mineralocorticoid receptors (MR) and the glucocorticoid receptors (GR) (*Reul and De Kloet., 1985*). These receptors are widely expressed in the body and profusely co-expressed in the neurons of limbic structures (*Herman et al., 1989*). MR has higher affinity (tenfold difference) for glucocorticoids than GR, thus MR is activated at basal levels and GR at stress levels. In the brain, MR functions coordinately with GR in the control of stress and its impact on affective and cognitive function (*De Kloet et al., 2005, Herman et al., 2016*). Both receptor types can mediate fast nongenomic steroid effects through classical cell signaling pathway, but they are best understood as ligand-activated transcription factors.
MR is involved in the evaluation process and the beginning of the stress response. GR, which is only activated by large amounts of glucocorticoids, terminates the stress reactions, mobilizes the energy resources required for this purpose and facilitates recovery. GR promotes memory storage in order to be prepared for future events. Therefore, the two-stress systems form interacting signaling networks that underlie adaptive processes ranging from the appraisal of an unfamiliar stimulus to memory storage and retrieval. Notably, these two receptors also participate in various aspects of the control of energy metabolism, from appetite and macronutrient choice to energy disposition and storage (*De Kloet et al., 2005*).

4. BRAIN REGIONS SENSITIVE TO STRESS

4.a. Hippocampus

The hippocampus is the principal target site in the brain for adrenocortical steroids, as it has the highest concentration of receptor sites for glucocorticoids (*McEwen et al., 1969; McEwen 1982*). The hippocampus plays a role of feedback of the HPA axis during stress. Indeed, the stimulation of the hippocampus induces a reduction of glucocorticoids release (*Dunn and Orr, 1984*). The glucocorticoid feedback process implies mainly the subiculum of the ventral hippocampus. The glutamatergic neurons project to GABAergic interneurons expressed in hypothalamic (mPOA, DMH, periPVN) and medial forebrain (BST) structures that inhibit the PVN (*Herman et al., 2005*). One of the other main features of the hippocampus is its high neuroplasticity. Indeed, repeated restraint stress produces significant remodeling in CA3 pyramidal neurons (*Magarinos et al., 1996*; *Sousa et al., 2000*). These dendritic morphological changes are characterized by a reversible shortening and debranching of apical

dendrites (*Conrad et al., 1996*; *McEwen, 1999*). Hence, the hippocampus is one of the main brain regions involved in the stress response and displays a high neuronal structural plasticity.

4.b. Amygdala

CRH neurons of the hypothalamus are mainly regulated by the hippocampus and the amygdala. Well known for its critical role in fear responses, the amygdala has emerged as another key region of the brain in the modulation of stress responses. Basolateral amygdala process sensory information from the thalamus that are relayed to neurons in the central nucleus. The central nucleus of the amygdala becomes active and project to the BNST that contains neurons that stimulate the hypothalamus and activate the stress response. In contrast to the hippocampus that suppress the HPA system, the amygdala stimulates the HPA axis and the stress response (Figure 3).

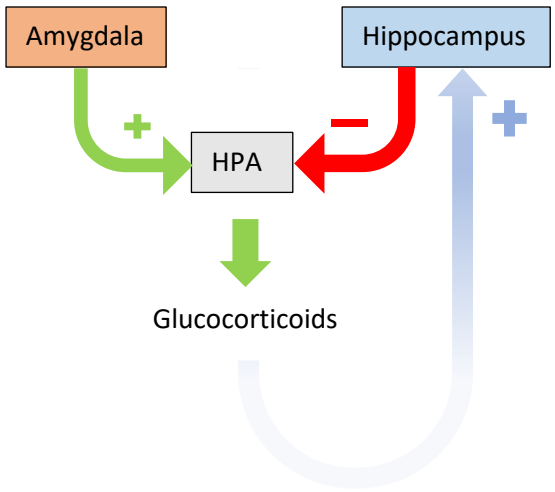

Figure 3. Cross-regulation of the HPA axis by the hippocampus and the amygdala

4.c. Prefrontal cortex

The amygdala presents a special connection with the prefrontal cortex (PFC). The PFC-the most evolved brain region- regulates our thoughts, actions and emotions through extensive connections with other brain regions. Acting with the amygdala, the PFC regulates emotion in adults (*Banks et al., 2007*; *Hariri et al., 2003*) and helps to evaluate the danger of an event. Hence, the prefrontal cortex plays a primary role in translating stressful emotional information into action. The PFC displays an elevated expression of GR that made this brain region highly sensitive to stress. A common task used to evaluate PFC cognitive function is the spatial working memory task. Rats and monkeys display an impairment of the spatial working memory after a mild acute stress (*Murphy et al., 1996; Shansky et al., 2006*). PFC seems to be particularly sensitive to architectural changes induced by chronic stress compared with other brain regions. While the hippocampus needs several weeks of stress exposure to show

neuronal structural changes (*McEwen et al., 2004*), dendrites in the PFC begin to change after only one week of stress (*Brown et al., 2005*) or possibly even a single exposure (*Izquierdo et al., 2006*).

5. STRESS AND SEX DIFFERENCES

Dysregulation of stress neurocircuitry is the most common feature across neuropsychiatric diseases, with both hyper- and hypo-reactivity of the HPA stress axis being reported (*Martin et al., 2010; Bale 2015*). Across lifespan men and women display a different prevalence for stress-related disorder. Epidemiological studies consistently show that women display a two-fold higher risk for any anxiety-related disorder and depression compared to men (*Breslau et al., 1997*; *Kessler et al., 2005; Altemus et al., 2014*). At the contrary, men are more susceptible de develop antisocial behavior or substance abuse than women (*Weich et al., 2001)*. Moreover, female victims of traumatic events are at higher risk for posttraumatic stress disorders than male victims (*Breslau, 2009*). Interestingly, the higher risk to develop affective disorders for women arise during the hormonally dynamic period called puberty pointing out the role of sex hormones in stress-related disorders (*Rubinow and Schmidt, 2019*). Sex differences are also present in the HPA axis. Studies in rodents reported sex differences in the HPA axis in response to acute stress. When rodents are in presence of physical and psychological stressors, females secrete higher absolute concentrations of corticosterone than males. Notably, this higher corticosterone concentration is observed basally and following a 30min restrain stress (*Aloisi et al., 1998*; *Mitsushima et al., 2003).* Moreover, both predictable and unpredictable stressors result in greater corticosterone release in females (*Heinsbroek et al., 1991)*. Females respond also better than males 5min after stressor onset because corticosterone increases more rapidly in females than in males (*Kant et al., 1983*) and levels remain elevated for a longer period (*Heinsbroek et al., 1991).* In human, the literature concerning sex differences in the stress response is less consistent than rodent studies because of the use of different methods, the diversity of age, health status and origin of subjects. Studies reported no difference or greater HPA activation in women. After exhaustive physical exercise no difference in cortisol has been reported (*Fridedmann et al., 1989*), however, another study showed that women display plasma ACTH and cortisol level higher than men following 1h of cold exposure (*Gerra et al., 1992*). The HPA axis in females seem to initiate more rapidly and produces a greater output of stress hormones compare to males. Because males and females display a different stress response, the inclusion of sex as a biological

variable in stress research is necessary for improving our understanding of disease mechanisms contributing to risk and resilience.

The brain is particularly sensitive to stress during specific period across lifespan. Chronic exposure to stress hormones, whether it occurs during the prenatal period, infancy, childhood, adolescence, adulthood or aging, has an impact on brain structures involved in cognition and mental health (*Lupien et al., 2009*; *McEwen et al., 2012*).

II. AGING

1. AGING HALLMARKS

Aging is characterized by a progressive loss of physiological integrity, leading to impaired function and increased vulnerability to death. This deterioration is the primary risk factor for

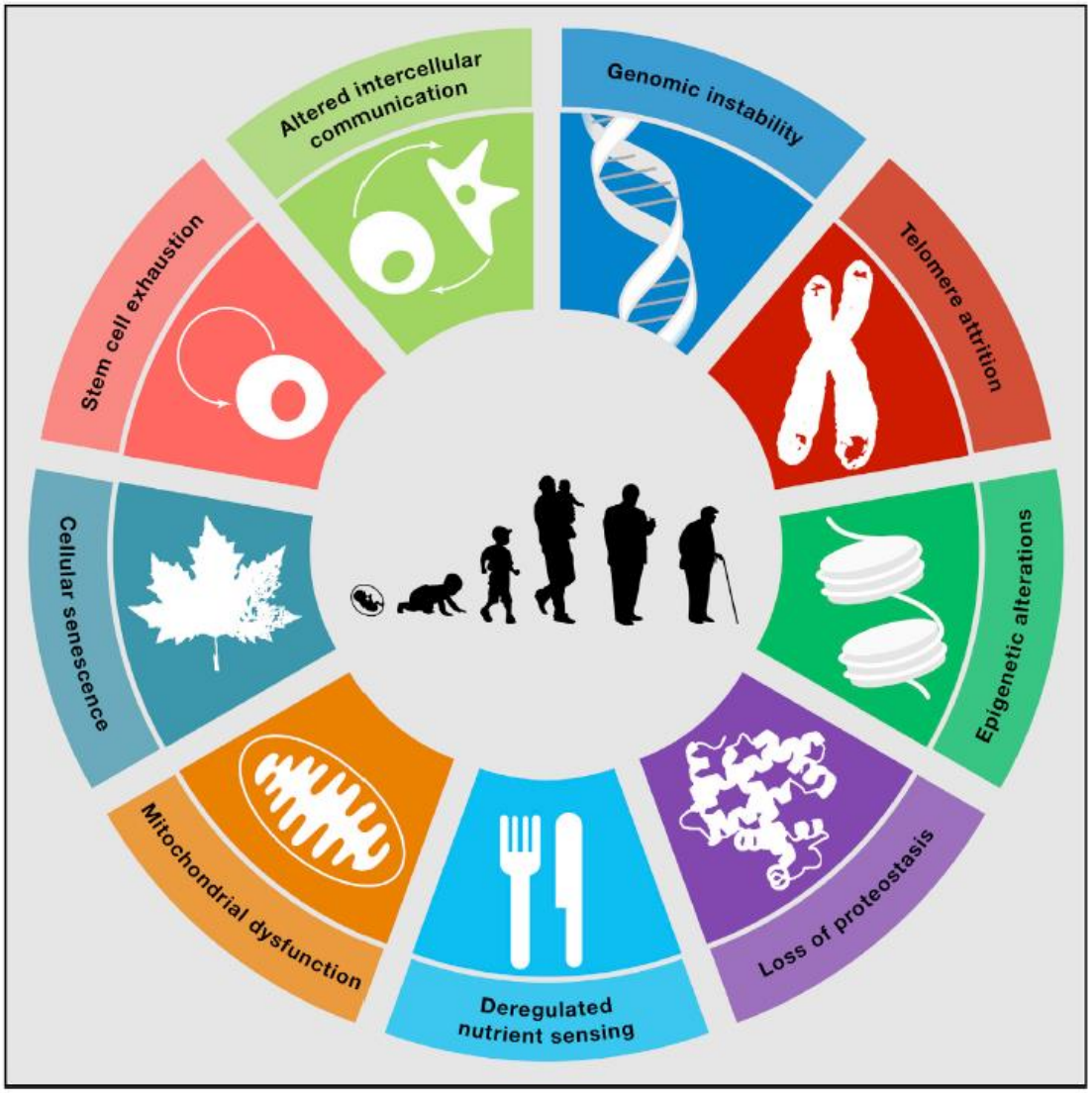

Figure 4. The hallmarks of Aging (from *Lopez-Otin et al., 2013)*

principal human pathologies such as cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases. Until now, several hallmarks of aging have been found on mammalian aging. These hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (*Carlos Lopez-Otin et al., 2013*).

A common characteristic of aging is genetic damages that accumulate throughout life (*Moskalev et al., 2012*). Accordingly, mutations and deletions in aged mtDNA contribute to aging (*Park and Larsson, 2011*) as well as defects in the nuclear lamina that can cause genome instability (*Dechat et al., 2008*). Aging causes telomere (*repetitive nucleotide sequences at both chromosome extremities*) exhaustion characterized by shorter telomere throughout normal aging in both human and mice (*Blasco et al., 2007*) and by telomere attrition. Importantly, stimulation of telomerase can delay aging in mice. Epigenetic alteration including histone modifications, chromatin remodeling, DNA methylation, constitutes another hallmark of aging. It has been shown that epigenetic marks such as H4K16 acetylation, H4K20 trimethylation or H3K4 trimethylation are increased during aging (*Fraga and Esteller, 2007*; *Han and Brunet, 2012*). Other evidence suggest that aging is associated with perturbed proteostasis, Indeed, perturbation of proteostasis can precipitate age-associated pathologies and improving proteostasis may delay aging in mammals (*Zhang and Cuervo, 2008*). The deregulation of nutrient sensing including the Insulin IGF-1 pathway as well as mTor, AMPK and Sirtuins is also considered as a hallmark of aging. The idea is supported by evidence showing that anabolic signaling accelerates aging and decreased nutrient signaling extends longevity (*Fontana et al., 2010*). Mitochondrial dysfunction is one of the most known aging marks and is characterized by deficiency of the respiratory chain that diminish with age and lead to electron leakage and a decrease of ATP generation (*Green et al., 2011*). The theory of mitochondrial free radical states that progressive mitochondrial dysfunction that occurs with aging results in increased production of ROS, which in turn causes further mitochondrial deterioration and global cellular damage (*Lopez-Otin et al., 2013*). The decline in the regenerative potential of tissues is one of the most obvious characteristics of aging. For example, hematopoiesis decreases with age leading to a diminution of adaptive immune cells production (*Shaw et al., 2010*). Similarly, stem cells attrition has been found in essentially all adult stem cell compartments, including the mouse forebrain (*Molofsky et al., 2006*). However, there is hope because of the reversal of stem cell aging at the cellular level might lead to the rejuvenation of the animal at an organismic level and the prevention of aging

(*Randoand Chang, 2012*; *Neves et al., 2017*). Aging also induces changes at the level of intercellular communication, be it endocrine, neuroendocrine, or neuronal (*Laplante and Sabatini, 2012*; *Rando and Chang, 2012*). Notably, neurohormonal signaling including adrenergic, insulin-IGF1 signaling tends to be deregulated in aging suggesting that altered intercellular communication is another aging hallmark .

Understanding aging process is essential to push the limits of human lifespan but also, and maybe more importantly nowadays, the life quality of the aging population.

2. STRESS AND AGING

Stress is an integral part of individuals 'life. Stressful experiences shape individuals. The relation between stress and aging has already been suggested in 1950 by Hans Selye stipulating that "*every stress leaves an indelible scar, and the organism pays for its survival after a stressful situation by becoming a little older"*. Several evidences reinforced the idea that chronic stress could accelerate aging process. Different hypotheses have been suggested to explain the relation between aging and the stress response. One of the most well-known is the "glucocorticoid cascade hypothesis of aging" (*Sapolsky et al., 1986*). The idea that glucocorticoids could promote brain aging originally came from studies showing a positive correlation between biomarkers of hippocampal aging and plasma corticosterone in aging rats. Landfield and colleagues selected astrocyte reactivity as an aging biomarker in hippocampus and found that it increased steadily with aging, being apparent even by mid-life. The increasing astrogliosis was correlated quantitatively with plasma corticosterone and adrenal weight, which also increased by mid-life. Moreover, they found that the exposition of middle-aged rat to high levels of exogenous glucocorticoids for a long period led to the development of hippocampal atrophy and memory impairment (*Landfield et al., 1978*a, b). Following these pioneer studies, Sapolsky, McEwen and collaborators found that hippocampal glucocorticoid receptors were reduced during aging with loss of negative feedback explaining the elevation of glucocorticoids and its neurotoxicity (*Sapolsky et al., 1986*; *Sapolsky et al., 1988*) These data provided extensive support for the view that glucocorticoids receptors were elevated with aging and could damage the hippocampus or increase its vulnerability. More recently, a causal relationship has been established between longevity genes expression in the hippocampus such as sirtuin-1, NADPH oxidase, p53 and chronic stress (*Sanchez-Hidalgo et al., 2016*).

In human, a reduction of telomere length has been found in individuals with depression or Post-traumatic stress disorders (*Zhang et al., 2014*). Further human studies revealed that elevated plasma glucocorticoid levels over years in older adults negatively correlates with

hippocampal volume and memory (*Lupien et al., 1998*). Interestingly, individuals with Alzheimer disease display memory impairment, hippocampal atrophy and higher basal glucocorticoid levels compare to controls (*Giubilei et al., 2001).* Furthermore, a randomized controlled trial of chronic glucocorticoid treatment (prednisone) has been shown to worsen cognition in people with Alzheimer's disease (*Aisen et al. 2000)*

All these data highlight the tight relation between chronic stress and aging mediated, in part, by the deleterious effect of glucocorticoids. Sex differences can be found at all stages of life and therefore influence the longevity of individuals.

3. SEX DIFFERENCES, AGING AND STRESS

Men and women are not equal in the face of aging. Indeed, one of the most potent characteristics of human biology is women's superiority in survival and longevity (*Austad 2019*). Women display a higher longevity than men around the world independently of culture or socio-economic status (*Zarulli et al., 2018*). Female longevity is also observed in the animal kingdom (*Austad and Fisher, 2016*). More specifically, most studies in rats found that female rats live longer than males ranging from 2% to 15% of survival advantage rate in females (*Austad 1997; Swindell, 2012; Turturro et al., 1999*). One of the biggest questions of this field is to find the mechanisms that can explain these sex differences in longevity. It is well known that sex chromosomes or gonads cause intrinsic sex differences in mammals and therefore could explain sex differences in longevity. In order to test whether they directly contribute to increased female lifespan, Davis and colleagues used a genetic manipulation that generates XX and XY mice, each with either ovaries or testes. They found that XX mice with ovaries or testes lived longer than XY mice of either gonadal phenotype, indicating a main effect of sex chromosomes on lifespan. Furthermore, mice with ovaries (XX & XY) tended to live longer than those with testes (XX and XY), suggesting a gonadal influence on lifespan. Carrying XX sex chromosome and ovaries extends lifespan in mice in mice highlighting that sex chromosomes and gonads influence mice longevity (*Davis et al., 2019*).

During aging, the brain is also at risk to the effects of stress, where the rapid decline of gonadal hormones in women in addition with cellular aging processes build up sex biases in stress dysregulation. Knowing the important roles of reproductive hormones on stress neural circuitry and HPA axis function, it is not surprising to see that women are two to three times more likely to experience a first episode of depression during the perimenopausal period, and the onset of schizophrenia after age 40 is two times higher in women than in men (*Nemeroff,*

2007 ; *Freeman et al., 2014*). Gonadal hormones impact brain structure, synaptic plasticity and are critical for executive function, learning and memory, and stress regulation. Hence, gonadal hormones can promote sex-specific outcomes during aging (*Shanmugan and Epperson, 2014*). Sex hormones influence the HPA axis as revealed by animal studies showing that aged female rats have higher basal corticosterone levels compared to same age males (*Bowman et al., 2006*). Moreover, testosterone reduces global corticosterone levels in response to a stressor and affect vasopressin as well as CRH synthesis and release (*Viau, 2002*). Thus, a reduction in testosterone in the brain in aging males would result in an increased stress response. On the other hand, estrogen is vital for neural plasticity because this hormone affects hippocampal and hypothalamic synaptogenesis and neuronal membrane firing potential (*Woolley, 2007*). Hence, a decrease of estrogen, notably during menopause transition in women can alter cellular processes involved in HPA axis activation and feedback.

Taken together, these studies strengthen the crucial role of gonadal hormones in the modulation of stress responsivity across the lifespan, and further suggest the influence of rapidly declining hormone levels in aging on how the brain perceives and responds to stress, especially in women (*Bale and Epperson, 2015*).

4. DOPAMINE

4.A. Dopamine and aging

Aging is associated with prominent declines in multiple components of the brain's dopamine system which have been linked to disrupted cognition, executive and motor functions (*Erixon-Lindroth et al., 2005*; *Li et al., 2020*; *Reeves et al., 2002*). Aging is well known to be the main risk factor for neurodegenerative pathologies including Parkinson's disease (PD) and Alzheimer's disease (AD) and alterations of the dopaminergic system have been linked to Alzheimer and Parkinson diseases. Nobili and colleagues found an age-dependent dopaminergic neuron loss in the ventral tegmental area (VTA) in a mice model of Alzheimer disease (*Nobili et al., 2017*). In Parkinson's disease, motor symptoms result from the loss of dopaminergic neurons in the substantia nigra (*Kalia and Lang 2015*).

Dopaminergic synapse

Dopamine is a neurotransmitter that is well known for its key role in reward system and motivation but is also involved in cognitive, motor functions and memory. In the central nervous system, dopamine-producing neurons are predominantly concentrated in the substantia nigra (SN) and the ventral tegmental area (VTA). Dopamine (DA) terminals are

widespread in the brain, dominating in the hippocampus, prefrontal cortex, striatum, and olfactory bulbs (*Beaulieu et al., 2011)*). Dopamine is synthetized in neurons from tyrosine. Tyrosine hydroxylase transforms tyrosine into DOPA and DOPA is synthetized into dopamine by DOPA-decarboxylase. Dopamine is then charged into synaptic vesicles via VMAT-2 (Vesicular monoamine transporter 2) and released into the synaptic cleft. In the extracellular space, dopamine can bind its post-synaptic receptors that induce a post-synaptic signal. Importantly, most of the dopamine released is recaptured by dopaminergic neurons via the dopaminergic transporter (DAT) (*Rice et al., 2011*).

Dopaminergic receptors are G-protein coupled receptors divided in two class: D1 and D2-class receptors. They share a high level of homology of their transmembrane domains and have distinct pharmacological properties. D1-class receptors include D1 and D5 that are coupled to Gs protein and are found exclusively at the post-synaptic level. Functionally, they activate the adenylate cyclase (AC) that stimulate cAMP production that lead, usually, to an increase of the neuronal excitability. At the contrary, D2-class receptors include D2, D3 and D4, are coupled to Gi protein and thus induce inhibition of AC. D2 and D3 dopamine receptors are expressed both post-synaptically on dopamine target cells and presynaptically on dopaminergic neurons (*Sokoloff et al., 2006*; *Rankin et al., 2010*; *Rondou et al., 2010*). In the central nervous system, dopaminergic receptors display different brain region localization. For example, D1 and D2 are both expressed in the striatum but not D3, D4, D5. Moreover, D4 and D5 can be found in the hippocampus at the contrary of D1, D2 and D3 revealing a heterogeneity of dopaminergic receptor along their brain region localizations (*levey et al., 1993*; *Bouthenet et al., 1991*).

4.B. Stress and dopamine

Stress directly influences several basic behaviors which are mediated by the dopaminergic system such as sensorimotor functions, incentive motivation, reward processing, and reinforcement learning (*Pani et al., 2000; Cabib and Puglisi-Allegra, 2012*; *Ljungberg et al., 1992*). Furthermore, it is becoming evident that the dopamine system plays a key role in the stress response, in particular, in the pathological response observed in many psychiatric disorders (*Belujon and Grace, 2015*). A link between stress and the dopaminergic system could be the glucocorticoids that increase tonic VTA excitability. Moreover, after a transient rise in excitability following chronic stress, VTA-DA neurons degenerate (*Douma and De kloet, 2020*) showing the deleterious effect of high levels of glucocorticoids on VTA-DA neurons.

Stress affect also the function of the motor system in both human (*Maki & McIlroy, 1996*) and rats (*Metz et al., 2001, 2005*) and a comorbidity between motor and stress-related disorders has been found (*Laureti et al., 2016*; *Suguma et al., 2016*). With aging, motor performance decline (*Vanden Noven, 2014, Rantanen et al., 1998*). Age-related changes in the basic functional unit of the neuromuscular system, the motor unit, and its neural inputs have a profound effect on motor function.

The striatum is a critical component of the motor and reward system. Dopaminergic neurons from the substantia nigra project to the striatum that serves as the primary input to the rest of the basal ganglia; a group of subcortical nuclei. Several lines of evidence suggest that stress impairs the dopaminergic system in the striatum. For example, mice exposed to a temporary reduction of food (12 days) availability display a reduction of striatal D2 receptors, a known marker of addiction-associated aberrant neuroplasticity, as well as liability to relapse into maladaptive stress coping strategies (*Campus et al., 2017*). Other studies shed the light of the impact of stress on motor functions. Smith and colleagues showed that chronic stress and elevated corticosterone levels exaggerate motor deficits and neurodegenerative events in a Parkinson's disease rat model (*Smith et al., 2008*). A follow-up study showed that an antagonist treatment blocking MR or GR prevent motor impairments caused by acute restraint stress or corticosterone treatment (*Jadavji et al., 2011*). Moreover, changes in the activity of the direct and indirect pathways of the striatal motor circuit have been reported after exposure to uncontrollable stress (*Clark et al., 2015*) as well as alteration in striatal DA levels in response to chronic stress in adult rats (*Ida et al., 1982*). These data strengthen the idea that stress and glucocorticoids influence motor functions across lifespan.

The capacity of an individual to cope with stress during across lifespan is influenced by stressful events occurring during early life (*Maccari et al., 2014*; *Maccari et al., 2017*).

III. EARLY LIFE STRESS

1. DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

It is commonly known that the interaction between factors in our lifestyle, like diet, physical exercises, smoking, drugs and genetical factors determine the susceptibility to develop major disorders of adult life such as diabetes, stroke and heart disease. A less well-known factor but as important as the others, if not more, is the growth in utero. This concept arises in the end of the 1900s when several epidemiolocal studies showed that undernutrition during fetal growth

increase the susceptibly to develop major disorders during adult life. The fetal growth need oxygen and nutrient to develop properly. When the intrauterine environment is poor in nutrient, meaning in undernutrition, the fetus adapts in order to sustain its development. This adaptation results in a low birthweight in human. Low birthweight has been linked with coronary heart disease in adult life (*Barker and Osmond, 1986, Barker et al., 1993*) pointing out a possible programming of coronary heart disease from poor intrauterine environment. Moreover, low birthweight was also associated with the insulin-resistance syndrome and its associated disorders later in life (*Barker et al., 1993*), as well as raised blood pressure in childhood and adult life (*Barker et al., 1990*) showing that fetal undernutrition is linked to hypertension in humans. Numerous animal experiments also showed that undernutrition in utero leads to persisting changes in blood pressure, cholesterol metabolism, insulin response to glucose, and a range of other metabolic, endocrine and immune functions known to be important in human disease (*Barker et al., 1997, Lucas, 1991*). This plethora of epidemiological studies gave rise to the concept of the intrauterine programming of adult disease (*Barker et al., 1995*) more commonly called the Developmental Origins of Health and Disease (DOHaD).

2. EARLY LIFE STRESS PROGRAMMING

Programming denotes that an environmental factor acts during a sensitive developmental period to affect the development and subsequent organization of specific tissues, which produces effects that persist throughout life (*Seckl et al., 2007, Weinstock. 2008*). As explained before, fetal malnutrition programs the mammalian fetal but this is not the only major environmental hypothesis that has been proposed. Indeed, an overexposure of the fetus to glucocorticoids or stress is also a major environmental hypothesis that have been proposed to underlie mammalian fetal programming. Offspring from pregnant dams submitted to prenatal restraint stress during the last week of gestation display increased plasma corticosterone levels at 3 and 21 days after exposure to novelty. At 90 days, prenatally stressed rats showed a longer duration of corticosterone secretion when exposed to an acute stress. These alterations of the corticosterone secretion were associated with a decrease of glucocorticoid and mineralocorticoid receptors densities in the hippocampus of prenatally stressed rats at 21 and 90 days but not at 3 days (*Henry et al., 1994*). These data were confirmed by another study showing that prenatal stress prolongs the corticosterone secretion two hours after an acute stress in adult male rats. This long-lasting corticosterone secretion pic in prenatally stressed rat was associated with a diminution of corticosteroid receptors in

hippocampus (*Maccari et al., 1995*). Hence, the HPA axis is suggested to be one of the biological substrates of the long-term effects of certain perinatal events. Glucocorticoids are crucial during fetal development for the maturation of tissues and organs, promoting cellular differentiation, and most notably acting during late gestation to stimulate surfactant production by the lung. Meanwhile this action is critical to prepare the fetus for extrauterine life, an overexposure of the fetus to glucocorticoids has adverse effects (*Seckl et al., 2009*). In concordance with this idea, prenatal exposure to dexamethasone or betamethasone (synthetic glucocorticoids) reduces birth weight (*Benediktsson et al., 1993*) and adult cortico steroid-exposed offspring are characterized by hypertension, hyper insulinemia, hyperglycemia, hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis and affective disorders (*Seckl et al., 2004*).

During normal pregnancy, maternal glucocorticoid levels are significantly higher than those in the fetal circulation. In order to avoid a too high concentration of glucocorticoids the fetus is protected from the relatively high maternal glucocorticoid levels of pregnancy by the placental inactivation of active glucocorticoids to its inactive forms by the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11β- HSD2). This enzyme acts as a 'barrier' to prevent premature or inappropriate action at glucocorticoid-responsive tissues during fetal development.

Reduced placental 11 β -HSD2 in human pregnancy correlates with lower birth weight and higher blood pressure in later life (*Lindsay et al., 1996*; *Murphy et al., 2002*) and mutations in the HSD11B2 gene in humans, markedly reduce birth weight (*Dave-Sharma et al., 1998*). Furthermore, in animal models, inhibition or knockout of placental 11 β -HSD2 decreases offspring birth weight, in part by lowering glucose delivery to the developing fetus in late gestation (*Holmes et al., 2006*). Molecular mechanisms thought to underlie the programming effects of early life stress and glucocorticoids include epigenetic changes in target chromatin, notably affecting tissue-specific expression of the intracellular glucocorticoid receptor (GR). Hence, excess glucocorticoids in early life can permanently alter tissue glucocorticoid signaling effects which may have short-term adaptive benefits but increase the risk of later disease (*Seckl et al., 2009, Patrick O. McGowan et al., 2018).*

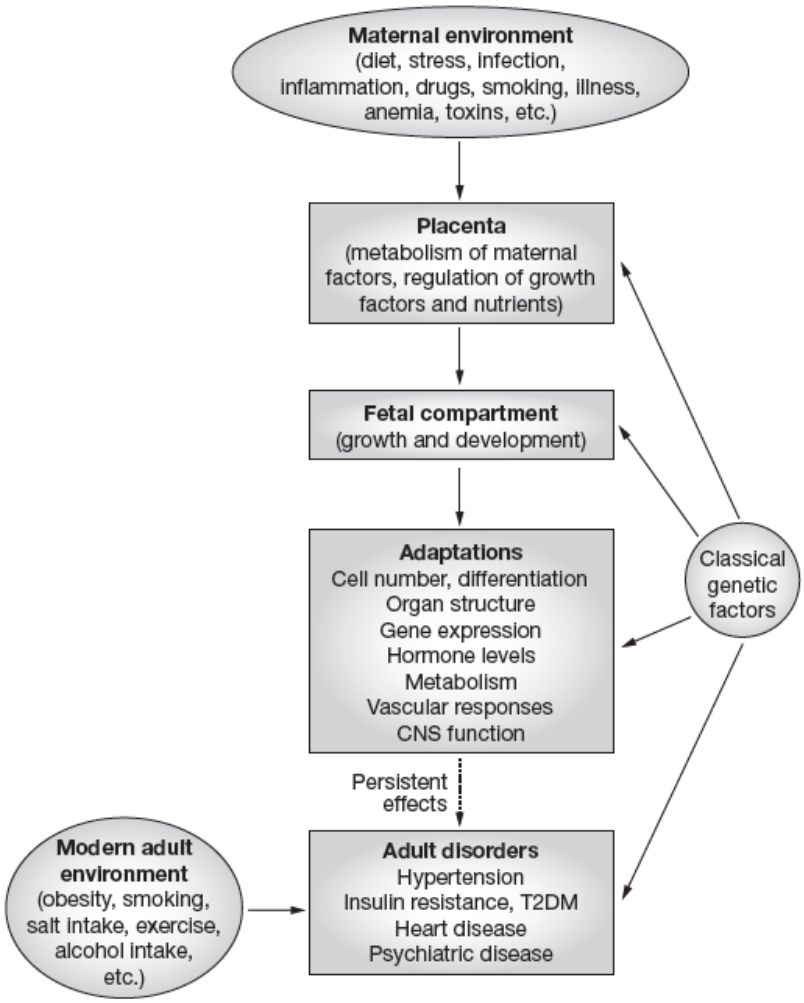

Figure 4. The concept of developmental programming. Maternal factors that affect the fetus during a short time-window can have long-lasting effects on exposed offspring, perhaps via epigenetic phenomena. In mammals this concept is exemplified by variable maternal environmental factors that modify placental function and consequently affecting growth and development in the fetus. These modifications result in alterations of cell structure, function and number. The effects of these modifications might be transient or persistent. In conjunction with classical genetic factors, these alterations might allow the fetus, and later the adult, to adapt to their predicted environment and benefit in evolutionary terms; however, in conjunction with modern environmental risk factors, the alterations might instead increase the risks of cardiovascular, metabolic, neurological and endocrine disorders in later life. Abbreviations: CNS, central nervous system; T2DM, type 2 diabetes mellitus. *From Seckl et al., 2007.*

3. MATERNAL CARE AND EPIGENETIC MECHANISMS

Another critical sensitive developmental time window is early days after birth when maternal care plays a key role. Pioneer work by Meaney and collaborators shed light on how maternal care (licking, grooming and arched-back nursing) in rodents can program stress response in the offspring. Liu and colleagues showed that variation of maternal care program the HPA axis. Indeed, offspring from mother with high maternal care display, during their adult life, reduced plasma adrenocorticotropic hormone and corticosterone responses to acute stress, increased hippocampal glucocorticoid receptor messenger RNA expression, enhanced glucocorticoid feedback sensitivity, and decreased levels of hypothalamic corticotropin-

releasing hormone messenger RNA (*Liu et al., 1997*). Moreover, variations in maternal care appears to be associated with the development of individual differences in behavioral responses to stress in the offspring such as "anxiety-like behavior" and the expression of fearfulness in rats (*Caldji et al., 1998*). Interestingly, cross-fostering experiment showed that pups from stressed mothers that are adopted by a mother not stressed display a reversal of the effects of prenatal stress (*Maccari et al., 1995*). Furthermore, adoption per se increases maternal behavior and decreases the stress-induced corticosterone secretion peak in the adult offspring reinforcing the concept of programming by the maternal care. It has been shown later that this "programming" by maternal care involves epigenetic modifications defined as "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence". There are several processes acting on the DNA, by modifying the transcription machinery without modify the DNA molecule itself.

Among these processes it is well known that the DNA methylation (*Hotchkiss, 1948*) and hydroxymethylation (*Kriaucionis et al., 2009*) act on the transcription machinery. DNA is wrapped around histone octamers to form nucleosomes-the unit of chromatin (*Nestler et al., 2016*). These histone proteins are modulated by a large set of chemical modifications such as acetylation (*Wade et al., 1997*) methylation (*Jenuwein, 2001*), phosphorylation ubiquitination (*Shilatifard, 2006*) and sumoylation (*Shiio et al., 2003*). These chemical modifications open or close the chromatin leading to the transcription or repression of specific gene and then influence the gene expression.

In 2004, Meaney and collaborators showed for the first time that increased maternal behavior as pup licking, grooming and arched-back nursing by rat mothers altered the offspring epigenome at a glucocorticoid receptor gene promoter in the hippocampus. DNA methylation (one of the core modifications of the epigenome) was significantly different in the offspring depending of the level of maternal care. These differences persisted into adulthood and were associated with altered histone acetylation and transcription factor (NGFI-A) binding to the GR promoter. A pharmacological approach using histone deacetylase inhibitor reversed the epigenetic differences observed previously highlighting a causal relation among epigenomic state, GR expression and the maternal effect on stress responses in the offspring (*Weaver et al., 2004).* These results have been also observed in humans where researchers examined hippocampi from humans who were exposed to child abuse. They compared the DNA methylation at the same NR3C1 promoter and showed that child abuse is associated with hypermethylation of several sites, including the NGFI-A recognition element that was implicated in targeting demethylation in response to maternal care licking and grooming in

rats. The changes in DNA methylation are associated with differences in expression of the NR3C1 receptor, linking child abuse and hippocampal regulation of stress responses (*Mc Gowan et al., 2009*). Since then, a plethora of studies showed convincingly that the epigenetic regulation of gene expression plays a key role in mediating the programming effects of stressful conditions during early development, with implications for neurobiological functioning and behavior in human (*Wadhwa et al., 2009*; *Champagne 2012*; *Champagne 2013*; *Klengel, et al., 2013*) as well in rodents (*Roth et al., 2009*; *Murgatroyd et al., 2009*).

4. TRANSGENERATIONAL PROGRAMMING

Meanwhile most of the studies on early life stress focused their attention on the detrimental effects in the first-generation offspring, new studies showed that the effects of early life stress can be transmitted to future generations via epigenetic mechanisms. The idea that environmental factors not only programs the individual that is directly exposed to them, but that this programming is also transmitted across one or more generations (*Dunn et al., 2011*) has become clearer these last years. Most of the behavioral alterations observed in the offspring submitted to a post-natal stress (maternal separation) were also found in the subsequent generation. Chronic and unpredictable maternal separation also alters the profile of DNA methylation in the promoter of several candidate genes in the germline of the separated males (*Franklin et al., 2010*). A treatment targeting the epigenetic marks can prevent the emergence of either emotional or cognitive deficits in the mother and also prevent the establishment of such deficits in offspring, indicating that transgenerational effects of early life stress can be prevented (*Schmauss et al., 2014).* Inducing a model of prenatal immune activation in mice, Weber-Stadlbauer and colleagues showed a reduced sociability and increased cued fear expression that were present in the first- and second-generation offspring. They further demonstrated that sensorimotor gating impairments are confined to the direct descendants of infected mothers, whereas increased behavioral despair emerges as a novel phenotype in the second generation showing that prenatal immune activation can negatively affect brain and behavioral functions in multiple generations (*Weber-Stadlbauer et al., 2017*). Moreover, studies in which female mice were exposed to nutritional challenges have provided some of the clearest examples of how phenotypes can be lost or retained across generations (*Dunn and Bale, 2009*; *Burdge et al., 2007*). While transgenerational effects of various changes in the maternal environment are well characterized, fewer studies have examined possible paternal modes of transmission. Paternal stress reduces HPA axis

responsivity in the offspring. This alteration of the HPA axis is associated with robust changes in sperm microRNA showing that paternal experience induces germ cell epigenetic reprogramming and impacts HPA axis (*Rodgers et al., 2013*). Using olfactory molecular specificity, another study showed that subsequent generations of mice subjected to odor fear conditioning displayed an increased behavioral sensitivity to the F0-conditioned odor. Further experiments revealed that the specific gene involves in this olfactory molecular pathway was hypomethylated in both F0 and F1 generations (*Dias and Ressler, 2014*). Furthermore, a recent study reported a transgenerational transmission of behavioral and metabolic phenotypes up to the 4th generation in a mouse model of paternal postnatal trauma (MSUS) (*van Steenwyk et al., 2018*).

These transgenerational studies highlight the idea that both maternal and paternal environment program the offspring and subsequent generations via the modulation of epigenetic marks.

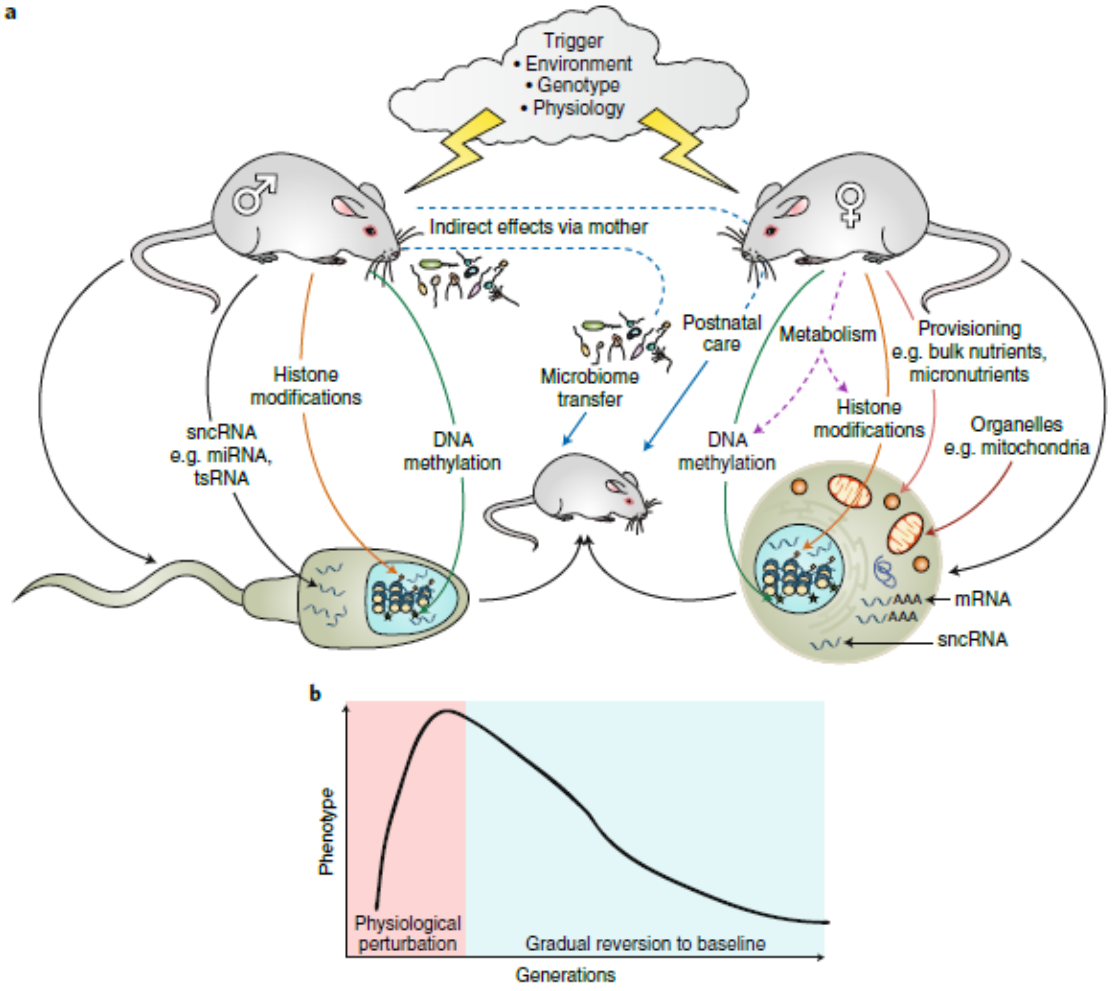

Figure 6. Mechanisms of transfer of information about ancestral environment or physiology over generations. **a**, Many mechanisms of transmission of information about environmental experience or physiological state can underlie inheritance over a single generation, from parents to

progeny, both genome associated (for example, covalent modifications of histones, sncRNAs, including tsRNAs and miRNAs, and DNA methylation, among others) and genome independent (for example, microbiome transfer). Paternal effects are not always mediated by gametes but may act via the mother indirectly. **b**, Gradual changes in epigenetic marks might underlie transgenerational memory. A loss of gene repression caused by an environmental or physiological insult, for example, by perturbation of heterochromatin-mediated transcriptional repression, can reset gradually over generations, providing a transgenerational memory of ancestral experience (From *Perez and Lehner, 2019*).

5. ANIMAL MODEL OF EARLY LIFE STRESS

In order to study the effects and the programming induced by the modification of the environment during early life, different animal model of early life stress has been developed. The first animal model of early life stress was set up in the middle of 1950s by Levine. This animal model of postnatal adversity consists of a daily separation of pups as a litter from the mother for 15 minutes from postnatal dal 1 to postnatal day 21 (*Levine 1957*). Following this, others animal model using handling procedure appeared. Maternal separation consisting of daily separation of the pups individually or as a litter from the mother for 3h or more from PND1 to PND14 was set up by Plotsky and Meaney (*Plotsky and Meaney, 1993*). George and collaborators developed a model of Maternal Separation with Early Weaning Model consisting of 3h of maternal separation between PND2 (post-natal day 2) and PND17 followed by early weaning at PND17. Interestingly maternal separation alone didn't induce any behavioral alteration in elevated plus maze or in the forced swim test maybe because of potential compensatory effects from maternal care (*George et al., 2010*). Another post-natal handling consists to remove the mother for 24h at PND9. This procedure is called maternal deprivation (*Roceri et al., 2002*). Every mother displays different maternal care to her pups. Hence mother with low maternal care and high maternal care can be differentiate knowing the time spent of licking, grooming and arched back nursing). Separating low maternal care versus high maternal care consists of another paradigm used to study postnatal environmental alterations (*Liu et al., 1997*; *Champagne et al., 2003*, *Weaver et al., 2004*). Besides models of postnatal stress, models of prenatal stress are also critical for the programming of a vulnerable phenotype in the offspring. Plenty of different kind of prenatal stress are commonly use nowadays such as undernutrition, infection, hormonal manipulation, physical stress, drug intake (*Bock and Braun, 2011*; *Muhammad and Kolb, 2011*; *Suenaga et al., 2012*; *Mychasiuk et al., 2011*; *Xu et al., 2013*). A rodent model of prenatal social stress has been raised by

Brunton and Russell. This model consists to expose pregnant rats to social stress by placing them into a cage with unknown lactating mother. The time of exposing is variable but is usually 10min/day form days 16 to 20 of pregnancy (*Brunton and Russell 2010*; *Brunton 2013*). These animal models are commonly used to study programming, to model stress-related disorders later in life as well as understand better involved in the long-term and transgenerational effects of prenatal stress and to develop and long-term effects and the potential strategies to adopt to cope with stress-induced diseases.

Very interestingly, the animal model that we used in our laboratory combines both prenatal stress and post-natal stress. The early-life stress procedure we use consists to submit pregnant dams to a restraint stress procedure (*Maccari et al., 1995*; *Mairesse et al., 2015*; *Marocco et al., 2012*; *Marrocco et al., 2014*; *Morley-Fletcher et al., 2018*; *Reynaert et al., 2014*).From day 11 of pregnancy until delivery, the dams are subjected to three stress sessions per day (45 min each during the light phase around 9:00 am, 12:00 pm, and 3:00 pm.), during which they are taken to a different room and placed in transparent plastic cylinders with conical end caps and exposed to bright light. At birth, pups were left undisturbed with their mother until weaning 21 days after birth. Mothers submitted to this restraint stress procedure display a low maternal care compared to unstressed mothers (control). Hence our animal model of early life stress integrates a pre- and post-natal stress and thus is defined as perinatal stress.

6. BEHAVIORAL CHARACTERIZATION OF EARLY LIFE STRESS RODENT ANIMAL MODEL

The face validity corresponds to the similarity in some mechanisms observed in human in the animal model. The construct validity represents similar causes between human and animals. Finally, the predictive validity corresponds to how a pharmacological treatment can be translated from human to the animal model (*Wilner., 1984; Berton., 2012*). Considering these three criteria, several lines of evidence showed that early life stress lead to decrease of the risk-taking behavior in the elevated plus maze, a reduction of the time spent in the center of the open field, a diminution of the time spent in the light space of the light/dark box test (*Zuena et al., 2008*; *Marrocco et al., 2012*; *Marrocco et al., 2014*). Altered risk-taking behavior observed in PRS rats is correlated to a decrease of the glutamatergic release specifically in the ventral hippocampus in adult rats and is reversed after increasing glutamatergic release via pharmacological approach (*Marrocco et al., 2012*). Furthermore, chronic treatment with two classical antidepressants corrects the defect in glutamate release and altered behavioral phenotype in PRS rats supporting the glutamatergic hypothesis of

stress-related disorders (*Marrocco et al., 2014*). Because studies suggest that early-life stress in rats predisposes to drug addiction (*Morley-Fletcher et al., 2004*; *Darnaudery and Maccari, 2008*; *Maccari et al., 2014*; *Galler and Rabinowitz, 2014, Reynaert et al.,2014*) animal models of early life stress are also used to study addictive behaviors. Indeed, it has been shown that early life stress enhances the vulnerability toward addiction (*Deminière et al. 1992*; *Morley-Fletcher et al. 2004*; *Kippin et al. 2008*; *VanWaes et al. 2009*). Moreover, early life stress has a profound impact on hedonic sensitivity to high-palatable food in a sex-dependent manner via long-lasting changes in gonadal hormones (*Reynaert et al., 2014*). Furthermore, studies on social interaction, locomotor activity, and prepulse inhibition showed that early life stress induces also a deficit in these behaviors associated with glutamate receptor activities (*Matrisciano et al., 2012*; *Matrisciano et al., 2013; Dong et al., 2015*; *Matrisciano et al., 2018*; *Nicoletti et al., 2019*).

7. EARLY LIFE STRESS EFFECTS ON AGING PROCESS

Besides to model neurological disorders, early life stress has long-lasting effects and alters different physiological parameters involved in aging process. Early-life stress induces long-term effects on the HPA axis characterized by the prolongation of the secretion of glucocorticoids following acute stress exposure in adult as well as in aged rats. Notably early life stress leads to cognitive deficit in aged rats but not in adult rats (*Vallee et al., 1999*; *Maccari et al., 1995*; *Henry et al., 1994*). Additionally of accelerating the HPA axis aging, early life stress accelerates aging process in adult rat depicted by a decrease of neurogenesis (*Lemaire et al., 2000*) and inflammation markers (*Vanbesien et al., 2007*) and an alteration of social memory and synaptic plasticity (*Marrocco et al., 2012*; *Marrocco et al., 2014*; *Brunson et al., 2005*). Another important characteristic of the anticipated aging triggered by early life stress is the decrease of sex hormones levels (*Van-Camp et al., 2018*; *Reynaert et al., 2014*) suggesting sex differences in early life stress responses.

8. SEX DIFFERENCES IN EARLY LIFE STRESS RESPONSES

The sexually dimorphic developing brain is dependent of developmental hormone exposure. Males face elevated testosterone levels during the process of normal testes development, mainly in utero, although the exact timing is species dependent. Aromatization of testosterone to estradiol in the brain drives masculinization, an active process affecting cell differentiation and connectivity in the brain in rodents (Figure 7; *McCarthy, 1994*; *McCarthy, 2016*). Hence

this is not surprising that early life stress occurring during critical time window for the sexual brain differentiation has different effects on males and females.

This is notably the case concerning neurodevelopmental disorders in human. Where studies have linked sex-biased neurodevelopmental disorders, including autism and schizophrenia, with fetal antecedents such as prenatal stress. Consistently with this idea, epidemiological studies showed that women exposed during their second trimester of pregnancy to the stress of the 1940 invasion of The Netherlands had male, but not female, children with an increased risk of schizophrenia (*Os and Selten, 1998*). A significant association between maternal psychosocial stress experienced during the first trimester of pregnancy has also been reported to significantly increase the risk of schizophrenia, once aging, only in male children (*Khashan et al. 2008*).

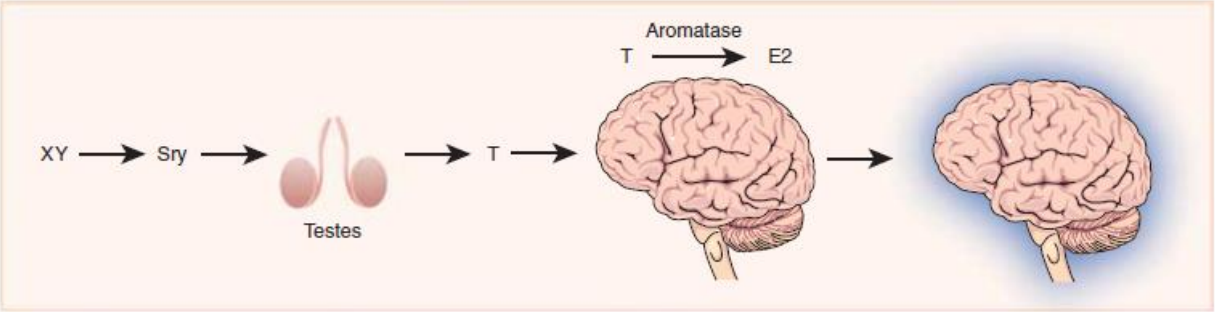

Figure 7. **Masculinization of the male brain.** In most mammalian species, testosterone is produced by the testes during testes development in late gestation. The presence of testes results from the testis-determining factor, Sry, on the Y chromosome that determines the direction the bipotential gonad develops. Circulating testosterone enters the brain and is aromatized to estradiol during this critical organizational window. Estradiol produces hosts of cellular effects to then 'masculinize' the male brain (*Bale and Epperson, 2017*).

Moreover, exposure to maternal stress before 32 weeks of gestation contribute to develop autism spectrum disorder (ASD), also a male-biased neurodevelopmental disorder (*Beversdorf et al. 2005*). Another study detected a significant effect of maternal depression during pregnancy on offspring postnatal anxiety development, an effect that was again significant only in boys (*Gerardin et al. 2011).* These studies suggest a higher sensibility in males to develop neurodevelopmental disorders and stress-related disorders following early life exposure and highlight the importance of fetal sex in the association between maternal stress and offspring long-term disease risk. (*Bale and Epperson, 2015*).

Sex is a critical factor in some behavioral phenotypes induced by perinatal stress (PRS) in rats, with males displaying higher susceptibility to emotional behavior in adult life (*Vallee et al., 1997, 1999; Zuena et al., 2008),* and reduced social play during adolescence (*Morley-Fletcher et al., 2003*). Moreover, PRS causes sex-dependent changes in hedonic sensitivity to highly palatable foods (milk chocolate) in adult life (*Reynaert et al., 2014*). Importantly, time exposure and type of early life stress leads to different effects as shown in this recent study where three different early life stress exposure paradigms impact differentially mast cell dynamics in the developing brain of male and female rats in hippocampus and hypothalamus (*Joshi et al., 2019*).

9. EARLY LIFE STRESS DISRUPTS MASCULINIZATION OF THE MALE BRAIN

Another particularly interesting point concern prenatal stress that feminizes and demasculinizes the behavior of males displaying a low level of copulatory behavior associated with low level of testosterone (*Ward 1972*). Prenatal stress in rats diminishes significantly the plasma testosterone values of male fetuses and newborns, so that the differences normally occurring between the two sexes is clearly reduced. This dysmasculinization of the brain in prenatally stressed rats was notably prevented by androgen administration in perinatal life (*Dorner et al., 1983*). More recently, Bale and collaborators showed that prenatal stress establishes sex differences by dysmasculinizing male offspring measures of stress responsivity (*Mueller and Bale, 2007*; *Mueller and Bale, 2008*) and epigenetically programs dysmasculinization in second-generation offspring via the paternal lineage (*Morgan and Bale, 2011*). Several line of evidences from our lab found a similar profile in perinatally stressed male rats. Indeed, PRS affects males to greater extent than females on the set-up of the circadian system, and cause dysmasculinization of PRS males related to locomotor activity phenotype as well as in gene expression in the central nervous system (*Morley-Fletcher et al., 2019*).

Globally, these few data provide evidence for a dysmasculinization profile induced by PRS at the behavioral and molecular level, highlighting the crucial need to include sex as a biological variable.

IV. NEUROPLASTICITY: GLUTAMATERGIC AND GABAERGIC SYNAPSES

1. NEUROTRANSMITTERS RELEASE

The human brain is composed of about 86 000 000 000 neurons (*Azavedo et al., 2009*; *Herculano-Houzel et al., 2012*). The incredible variety of functions performed by the brain is made possible by the ability of neurons to communicate with each other, which occurs primarily via chemical synaptic transmission. The synapse is composed of presynaptic and postsynaptic components. The presynaptic side of the synapse, usually an axon terminal, contains synaptic vesicles that store neurotransmitter. The arrival of an action potential triggers the neurotransmitter release in the synaptic cleft. This process involves neurotransmitters that are packaged in vesicles at presynaptic nerve terminals and are released by Ca^{2+}-evoked synaptic vesicle exocytosis. Proteins accumulate under the postsynaptic membrane that contains the neurotransmitter receptors. The activation of this receptor by the neurotransmitters convert a chemical signal into an intracellular signal in the postsynaptic cell. The neurotransmitters release depends of an exquisite and amazingly precise mechanism as we will discuss in the following part.

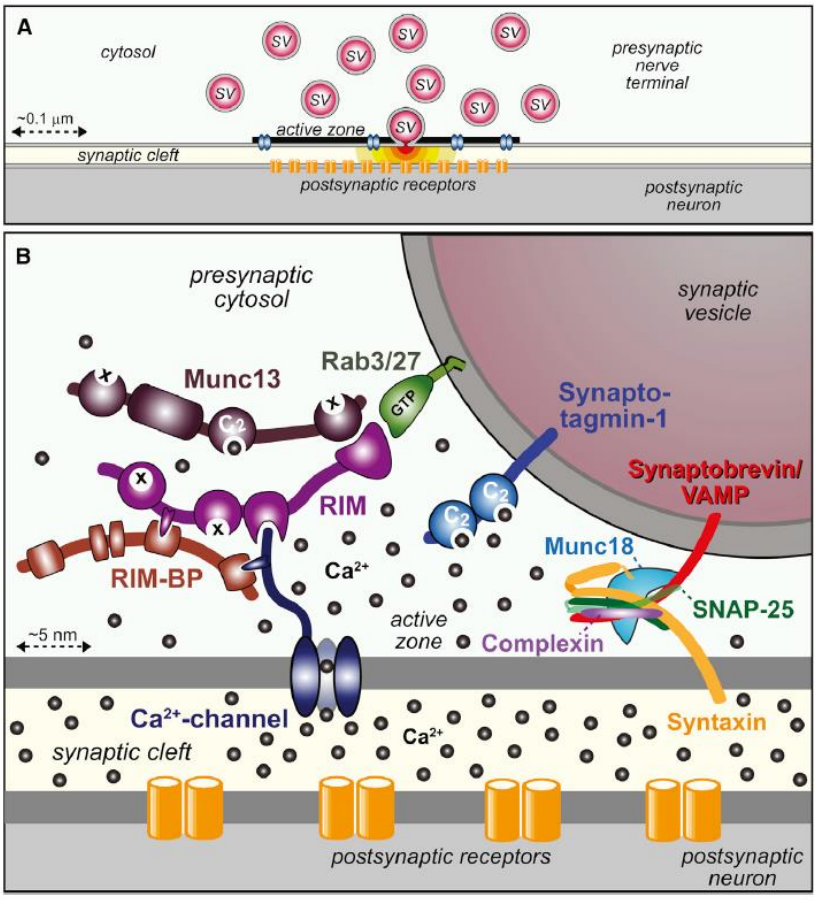

Figure 8. Organization of the Presynaptic Release Machinery (A) Drawing of a synapse with synaptic vesicles (SV, red), an active zone containing Ca2+ channels (blue), and a postsynaptic cluster of receptors (orange). One vesicle in the active zone is depicted in the process of fusing, with red neurotransmitters emitting from the fusion pore. (B) Schematic of the molecular machinery mediating Ca2+-triggered vesicle fusion. The drawing depicts a segment of a docked synaptic vesicle on the top right and the presynaptic active zone in the middle. The three functional elements of the neurotransmitter release machinery are depicted from right to left. On the right, the core fusion machine composed of the SNARE/SM protein complex is shown; this machine comprises the SNARE proteins synaptobrevin/VAMP, syntaxin-1, and SNAP-25 and the SM protein Munc18-1. The Ca2+ sensor synaptotagmin-1 is depicted in the middle; it is composed of a short intravesicular sequence, a single transmembrane region, and two cytoplasmic C2 domains that bind Ca2+, and it functions using complexin (bound to the SNARE complex) as an assistant. The active zone protein complex containing RIM, Munc13, and RIM-BP and a Ca2+ channel in the presynaptic plasma membrane is shown on the left. In this protein complex, RIM binding to specific target proteins coordinates all three functions of the active zone: RIM binding to vesicular rab proteins (Rab3 and Rab27 isoforms) mediates vesicle docking; RIM binding to the central priming factor Munc13 activates vesicle priming; and RIM binding to the Ca2+ channel, both directly and indirectly via RIMBP, recruits the Ca2+ channels within 100 nm of the docked vesicles for fast excitation-secretion coupling. The overall design of the neurotransmitter release machinery depicted here enables in a single nanodevice fast and efficient triggering of release in response to an action potential by combining a fusion machine with a Ca2+ trigger and an active zone protein complex that positions all elements into appropriate proximity (*From Sudhof 2013*).

The neurotransmitter release process begins with an action potential arriving at a presynaptic nerve terminal and gates Ca2+ channels. Ca2+ triggers the exocytotic fusion of synaptic vesicles containing neurotransmitters and the following neurotransmitter release in the synaptic cleft produce a postsynaptic signal (*Katz,1969*). The docking and the fusion of synaptic vesicles containing neurotransmitters are critical steps requiring the orchestration and functioning of the core fusion machinery composed of specific proteins.

This intracellular membrane fusion is mediated by SNARE (for ‘‘soluble NSF attachment receptor proteins’’) and SM (for ‘‘Sec1/Munc18-like proteins’’) proteins. The neuronal SNAREs involved in neurotransmitter release are syntaxin-1, SNAP-25 and synaptobrevin-2 (also called VAMP2). Synaptobrevin is referred to as a v-SNARE because of its vesicle localization, while syntaxin-1 and SNAP-25 are called t-SNAREs because they reside on the target membrane (*Sollner et al., 1993*). The underlying principle of SNARE and SM protein function is as follow. SNARE proteins embedded in the two fusing membranes form a trans-

complex that involves a progressive zippering of the four-helical SNARE complex in an N- to C-terminal direction (*Hanson et al., 1997*). Zippering of trans-SNARE complexes forces the fusing membranes into close proximity, destabilizing their hydrophilic surfaces. Assembly of the full trans-SNARE complex together with the action of the SM protein opens the fusion pore. Fusion pore expansion transforms the initial ‘‘trans’’-SNARE complexes into ‘‘cis’’-SNARE complexes that are then dissociated by NSF (which binds to SNARE complexes via SNAP adaptor proteins), completing the cycle (*Sudhof 2013*; *Sudhof 2012*). Importantly, the assembly of the SNARE complex provides the energy necessary for membrane fusion (*Sutton et al., 1998*). Since SNARE complex formation is not sensitive to Ca2+, other factors introduce the Ca2+ sensitivity that is required for synaptic vesicle fusion. Synaptotagmins are transmembrane proteins with two cytoplasmic C2 domains (*Perin et al., 1990, 1991*) that bind Ca^{2+} (*Brose et al., 1992*). Importantly, sixteen synaptotagmins are expressed in brain but only eight of which bind Ca2+. Among these eight proteins, synaptotagmin-1 (Syt1), which resides on the synaptic vesicle, appears to be vital for synchronous Ca2+-triggered synaptic vesicle fusion (*Fernandez-Chacon et al., 2001*; *Geppert M et al., 1994*). All synaptotagmin controlled fusion reactions appear to require complexin as a cofactor (*Reim et al., 2001*; *Jorquera et al., 2012*; *Cao et al., 2013*).

In summary, during an action potential, Ca^{2+} enters in a presynaptic terminal. Ca^{2+} binding to synaptotagmin triggers release by stimulating synaptotagmin binding to a core fusion machinery composed of SNARE and SM proteins that mediates membrane fusion during exocytosis. This process is assisted by complexin adaptors which activate and clamp this core fusion machinery.

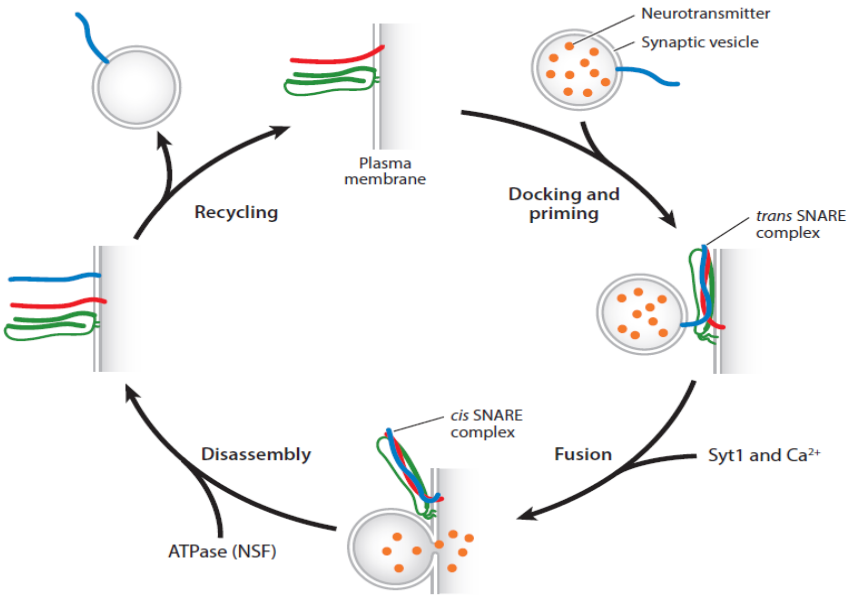

Figure 9. The roles of SNAREs in the synaptic vesicle cycle. SNAREs form a *trans* complex that juxtaposes membranes after synaptic vesicle docking and priming, and when it combines with Syt1, evoked fusion occurs upon an action potential. During fusion, SNARE complexes are fully formed (*cis* complex). *Cis* SNARE complexes are disassembled by the ATPase NSF in conjunction with SNAPs and made available for another round of synaptic vesicle formation. Abbreviations: NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF adaptor protein; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; Syt1, synaptotagmin-1 (*from Brunger et al., 2018*).

2. GLUTAMATE

Neurotransmitters are usually divided in three chemical categories: amino acids, amines and peptides. Glutamate is the most abundant excitatory neurotransmitter in the central nervous system. Glutamatergic neurotransmission occurs mostly within a tripartite synapse including presynaptic membrane and postsynaptic membrane surrounding by glia. There are several regulatory processes within the glutamatergic synapse including basal and stimulated presynaptic glutamate release; postsynaptic receptor trafficking and function; transporter-mediated uptake and recycling of glutamate through the glutamate–glutamine cycle (*Nicoletti et al., 2011*).

2.A. The glutamate-glutamine cycle

That cycle of neuronal-astrocytic interactions plays a major role in the metabolism of glutamate (*Berg and Garfinkel, 1971*; *Benjamin and Quastel, 1972*). Neuronal glutamate is both synthesized *de novo* from glucose via the TCA cycle (tricarboxylic acid cycle) and from glutamine (Gln) supplied by glial cells. Glutamate synthetized in the presynaptic terminal is packaged into synaptic vesicles by glutamatergic transporters (vGLUTs). As described previously, glutamate is released into the synaptic cleft and bind glutamatergic receptors expressed at the presynaptic membrane, postsynaptic membrane or glial cells. After that, glutamate is cleared from the synaptic cleft through excitatory amino acid transporters (EAATs) on neighboring glial cells (EAAT1 and 2). Glutamate is converted into glutamine via the glutamine synthetase pathway. Glutamine is then released into the extracellular space and taken up by presynaptic neurons and metabolized into glutamate by the phosphate-activated glutaminase completing the glutamate-glutamine cycle. This process between synaptic terminals and glial cells is essential in order to preserve a proper amount of glutamate (*Sonnewald and Schousboe, 2016*).

Glutamate, when released into the synaptic cleft, binds glutamatergic receptors which are divided into two categories: metabotropic glutamate receptors and ionotropic glutamate receptors.

2.B. Metabotropic glutamate receptors

The evidence that quisqualate and glutamate stimulated inositol phosphate formation in cultured striatal neurons (*Sladeczek et al.,1985*) offered the first demonstration that excitatory amino acids could activate receptors other than the classical ligand-gated ion Channels (ionotropic glutamate receptors). Meanwhile, ibotenic acid, an analogue of glutamate was found to enhance the hydrolysis of membrane inositol phospholipids in rat hippocampal slices suggesting the existence of a class of dicarboxylic amino acid recognition sites coupled with phospholipase C (*Nicoletti et al., 1986a,b*) called later by Sugiyama and collaborators metabotropic glutamate receptors (*Sugiyama et al., 1987*). The first metabotropic glutamate subtypes (mGlu1 receptor or mGluR1) was cloned and characterized by the team of Nakinishi (*Masu et al., 1991*) and led to the discovery of others metabotropic glutamate receptors. There are eight metabotropic glutamate receptors that has been identified until now. They are divided in three groups with group I including mGlu1 and mGlu5, groupII including mGlu2 and mGlu3, and group III including mGlu4, mGlu6, mGlu7, and mGlu8. mGlu1 and mGlu5 receptors are coupled to Gq/G11, whereas all other subtypes are coupled to Gi/Go (*Nicoletti et al., 2011*). mGlu receptors are G-protein coupled receptors (GPCR) that are membrane-bound proteins activating by extracellular ligands such as neurotransmitters and transduce intracellular signals via interactions with G proteins. The resulting change in conformation of the GPCR induced by ligand binding activates the G protein, which is composed of a heterotrimeric complex of α, β, and γ subunits. Activated G protein subunits modulate the function of various effector molecules such as enzymes, ion channels, and transcription factors (*Niswander and Conn, 2010*).

Structurally, these receptors have a large extracellular domain made of a Venus Fly Trap (VFT) domain linked to the first transmembrane domain (of 7 TM) via a cysteine-rich domain (CRD) containing 9 highly conserved cysteine residues (*O'Hara et al., 1993*; *Muto et al., 2007*). mGlu receptors are not only complex proteins because of their structural organization but also because they form dimers stabilized by an intersubunit disulfide bond (*Romano et al., 1996, Kunishima et al., 2000*). Until recently these receptors have been only considered as homodimers, but several studies revealed the possible existence of heterodimeric mGluRs (*Doumazane et al., 2011*; *Kammermeier, 2012*). Accordingly, metabotropic glutamate

receptors of the same group can form heterodimers and group-II and group-III mGlu receptors can form intergroup heterodimers. Among these heterodimers mGlu receptors, mGlu2-4 was the most studied pair due to its important physiological interest and has been observed in vitro as well as in native rat and mouse tissues (*Kammermeier, 2012*; *Niswender et al., 2016*). Importantly, no heterodimers can be formed between group-I and group-II/III mGlu receptors (e.g., mGlu1 and mGlu2 or mGlu4), suggesting that only mGlu receptor subtypes coupled to the same G protein can form heterodimers. mGlu receptors are broadly distributed throughout the CNS and are specifically localized at discrete synaptic and extrasynaptic sites in both neurons and glia in potentially every major brain region (figure 10). The metabotropic glutamate receptors participate in the modulation of synaptic transmission and neuronal excitability throughout the central nervous system. mGlu receptors from group I are often localized postsynaptically, and their activation usually leads to cell depolarization and to an increase of the neuronal excitability. Modulation of neuronal excitability results from modulation of several ion channels and can range from robust excitation to more subtle changes in patterns or frequency of cell firing or responses to excitatory inputs (*Conn and Pin, 1997*, *Anwyl, 1999*)). In contrast, group II and group III mGlu receptors are often localized on presynaptic terminals or preterminal axons where they inhibit neurotransmitter release. This occurs at excitatory (glutamatergic), inhibitory (GABAergic), and neuromodulatory (i.e., monoamines, ACh, peptides) synapses (*Niswander and Conn, 2010; Nicoletti et al., 2011).*

2.C. Ionotropic glutamate receptors

NMDA receptors

Glutamate acts on various membrane receptors, including ionotropic glutamate receptors (iGluRs), which form cation-permeable ion channel receptors and can be subdivided into three large families: NMDA receptors (NMDARs), AMPA receptors (AMPARs) and kainate receptors.

NMDARs have kept captivating neuroscientists because of their key roles in central nervous system function. These glutamate-gated ion channels are essential mediators of brain plasticity and are capable of converting specific patterns of neuronal activity into long-term changes in synapse structure and function that are thought to underlie higher cognitive functions. NMDARs are assembled as heteromers differing in subunit composition. Seven different subunits have been found. They are divided in three subfamilies following to

sequence homology: the GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D), which are encoded by four different genes, and a pair of GluN3 subunits (GluN3A and GluN3B) (*Traynelis et al., 2010*; *Cull-Candy and Leszkiewicz, 2004*; *Paoletti, 2011*). Importantly, subunit composition varies across CNS regions during development and in disease states (*Lau and Zukin, 2007*) (figure 11). Moreover, their subunit composition is essential because it determines receptor properties.

NMDARs have very special characteristics compared to other ionotropic receptors. The ion channel of NMDARs is subject to a voltage-dependent block by Mg^{2+}, NMDARs channel are highly permeable to Ca^{2+}, their activation requires the presence of glutamate and of glycine or D-serine. Well known for their crucial role in brain development and synaptic plasticity via the induction of long-term potentiation (The persistent strengthening of synaptic transmission) and long-term depression (A long-lasting decrease in synaptic strength), NMDARs are also found in presynaptic membrane where they may facilitate glutamatergic release. Importantly, NMDARs overactivating by glutamate allow high levels of Ca^{2+} to enter the cell. Calcium influx into cells activates different enzymes that damage the cell structures such as endonucleases, proteases and ultimately cause cell death. This process is called excitotoxicity. The dysfunction of NMDARs has been shown to be involved in multiple neurological and psychiatric disorder. (*Mohn et al., 1999*; *Gonzales-Burgos and Lewis, 2015*; *Tang et al., 2019*; *Tarabeux et al., 2011*). NMDARs is usually associated with AMPA receptors because of its crucial role is long-term potentiation and long-term depression.

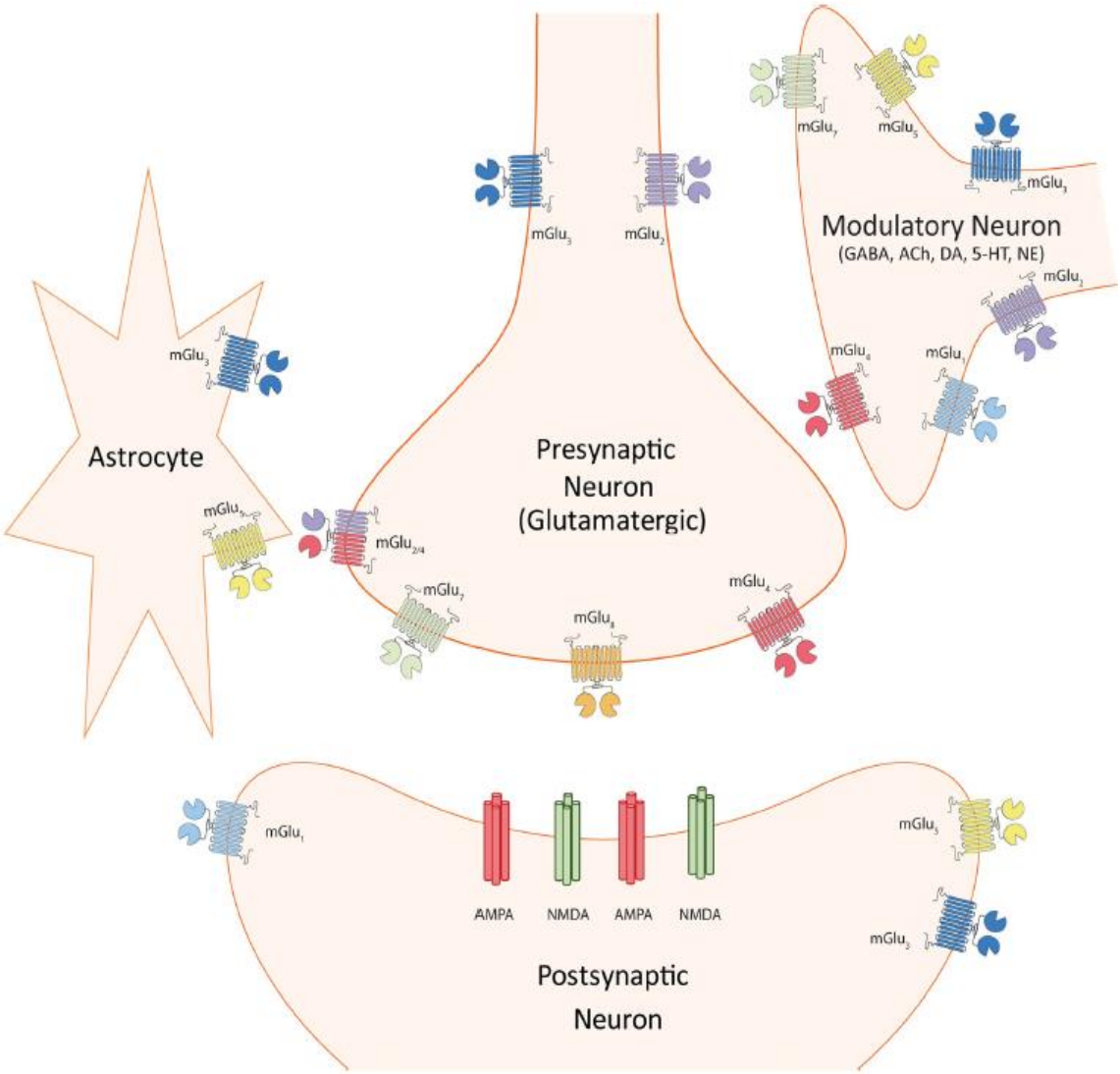

Figure 10. Localization of metabotropic glutamate receptor subtypes. Schematic representation of the predominant locations of mGlu receptors at the synapse. mGlu1 (light blue) is found on postsynaptic glutamatergic neurons as well as on GABAergic neurons. mGlu5 (yellow) can be located on the same neurons as mGlu1 as well as on glia. mGlu2 (purple) is found primarily presynaptically as both a homodimer as well as a heterodimer with mGlu4 (red). mGlu3 (dark blue) is found on both presynaptic and postsynaptic glutamatergic, GABAergic, and neuromodulatory neurons as well on glia. mGlu4 is localized to both modulatory neurons as well as on presynaptic glutamatergic neurons as either a homodimer or heterodimer. mGlu7 (green) is localized to presynaptically neurons as well as GABAergic neurons. Lastly, mGlu8 (orange) is primarily localized presynaptically and mGlu6 is not shown since it is restricted to the retina (*from Maksymetz et al., 2017*)

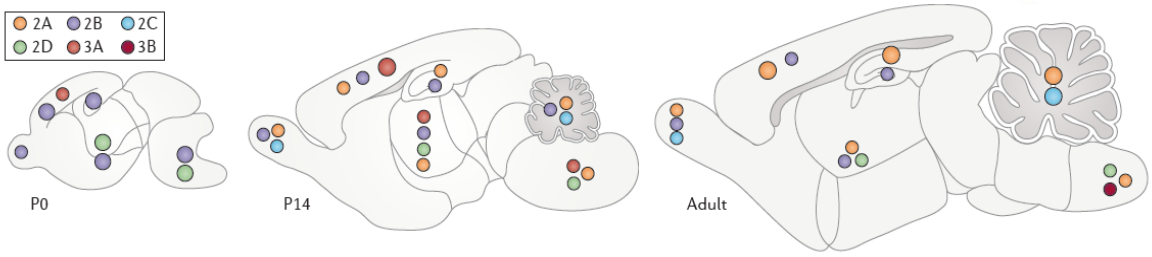

Figure 11. NMDAR subunit diversity during development. The developmental profile of GluN subunit expression in the mouse brain at day of birth (postnatal day 0 (P0)), 2 weeks following birth (P14) and at the adult stage (*From Paoletti et al., 2013*).

AMPA receptors

AMPARs are glutamate-gated ion channels permeable to Na^+ and K^+ that mediate most of the fast-excitatory synaptic transmission in the brain. AMPARs that are localized at the postsynaptic membrane are essential for excitatory synapse formation, stabilization, synaptic plasticity and neural circuit formation. Four subunits, GluA1–GluA4, assemble into tetramers to make up the core functional ion channel, with different combinations conferring unique cellular trafficking behaviors and biophysical characteristics (*Shepherd and Huganir, 2007*; *Diering and Huganir, 2018*). GluA1–GluA3 are expressed in the majority of neurons in the nervous system while GluA4 is primarily expressed early in life (*Zhu et al., 2000*). Changes in the properties and postsynaptic abundance of AMPARs) are major mechanisms implicated in synaptic plasticity. Along the type of synaptic plasticity (long-term potentiation or long-term depression) the function and the trafficking of AMPARs to and from synapses is modulated by specific AMPAR GluA1–GluA4 subunits, subunit-specific protein interactors, auxiliary subunits, and posttranslational modifications. Accordingly, Diering and Huganir proposed recently an AMPAR code where they hypothesize that AMPAR variants will be predictive of the types and extent of synaptic plasticity (*Diering and Huganir, 2018).*

Kainate receptors

Kainate receptors (KARs) are glutamate-gated ion channels that play fundamental roles in regulating neuronal excitability and network function in the brain. KARs are widely distributed throughout the brain and, depending on the cell type in question, they can be localized at pre-, post- and/or extrasynaptic sites. In general, presynaptic KARs modulate both excitatory and inhibitory neurotransmitter release, postsynaptic KARs contribute to excitatory neurotransmission and extrasynaptic KARs play a role in determining neuronal excitability (*Sihra TS, et al., 2014*; *Carta M et al., 2014*). There are five subunits of KARs named GluK1,

GluK2, GluK3, GluK4 and GluK5. They can be assembled in different ways in order to form a tetramer. Postsynaptic KARs are implicated in synaptic plasticity. Accordingly, activating postsynaptic KARs on CA1 neurons induce LTP of AMPARs via a metabotropic signaling pathway (*Petrovic MM et al., 2017*). Furthemore, KARs are involved in both excitatory and inhibitory neurotransmission. Indeed, presynaptic KARs decrease glutamate release at CA3-CA1 pyramidal cell synapses (*Chittajallu R et al., 1996*; *Frerking M et al., 2001*). However, activation of KARs with very low amount of kainate facilitates glutamatergic release (*Schmitz D, et al, 2001*; *Andrade-Talavera Y et al., 2013*) suggesting bidirectional modulalatory actions on glutamate (*Ashley J. Evans et al., 2019*). In addition to their role in excitatory neurotransmission, KARs has also been shown to be involved in the inhibitory neurotransmission where Presynaptic KARs downregulate GABA release from interneurons in the hippocampus via a metabotropic PKC and PLC dependent pathway reducing inhibitory postsynaptic currents (*Rodriguez-Moreno et al., 1998*).

2.D. Interactions between metabotropic and ionotropic glutamate receptors

Although glutamatergic receptors are subdivided in two groups, metabotropic and ionotropic glutamate receptor interact with each other. GluRs crosstalk occurs via functional and physical interactions. Scarce evidences suggest direct iGluR-mGluR interaction. Accordingly, BRET (Bioluminescence Resonance Energy Transfer) studies showed dynamic interaction between mGluR5 and NMDARs in HEK cells (*Perroy et al., 2008*) as well as in dendritic spines of hippocampal neurons (*Moutin et al., 2012*). iGluRs and mGluRs can also interact via synaptic scaffolding proteins that provide ample crosstalk opportunities by bridging different receptor complexes. Such interactions have been very well characterized between NMDARs and mGlu5 receptors that communicate via Homer-Shank-DLGAP-PSD95 (*Tu et al., 1999, Schwenk et al., 2012*). Instead of favorizing synergy between both classes of GluRs, the same scaffold protein can provide the possibility of competition. Such phenomenon has been observed for PICK1, a Ca^{2+} sensor (*Peter et al., 2004*), that contains a common binding site for mGlu7 receptors and AMPARs (*Hirbec et al., 2002*). Another potential mechanism of crosstalk between iGluRs and mGluRs is mediated by downstream signals that are activated by both iGluRs and mGluRs. Several works on GluRs mediated second messenger signaling crosstalk has focused on Ca^{2+} which is considered as the most important second messenger in the central nervous system because of its pivotal roles in presynaptic neurotransmitter release, postsynaptic responses, and plasticity induction (*Reiner and Levitz, 2018*). Glutamate-evoked

channel opening of NMDARs and a less extend KARS and AMPARs produces Ca^{2+} influx, whereas group I mGluRs (mGlu1 and mGlu5) induce Ca^{2+} release from intracellular stores. Both iGluRs and mGluRs can modulate the function of voltage-gated Ca^{2+} channels (VGCCs), and elevations in cytosolic Ca^{2+} can lead to Ca^{2+}-induced Ca2+ release (CICR) from the ER, providing many opportunities for crosstalk where Ca^{2+} signals act cooperatively to control cellular processes (*Reiner and Levitz, 2018*). Furthermore, iGluRs and mGluRs can interact also via the convergence of second messenger signaling such as kinases and phosphatases (*Lisman, J., et al., 2012*). GluRs crosstalk has been found to be involved in different neuropsychiatric disorders. One of the most striking examples is about the depression and the ketamine. This non-competitive NMDAR antagonist works as an antidepressant with rapid onset and long-lasting effects (*Duman et al., 2016*). Depression induces alteration of glutamatergic synapses mainly in the prefrontal cortex and ventral hippocampus. Accordingly, antagonism of either group I (*Hughes et al., 2013*) or group II (*Chaki, 2017*) mGluRs shows similar rapid anti-depressant effects in mouse models, suggesting overlapping mechanisms.

2.E. Early life stress impairs glutamatergic synapse

Alterations of synaptic transmission and plasticity in the hippocampus are an integral part of the abnormal programming triggered by early life stress. Early life stress reduces the glutamatergic release selectively in the ventral hippocampus of adult rats. Abnormalities of glutamatergic release was associated with large reductions of synaptic vesicle related proteins in the ventral hippocampus. The use of both mGlu2/3 receptor antagonist and $GABA_B$ receptor antagonist enhanced the glutamatergic release and abolished anxiety like behavior in perinatally stressed (PRS) rats suggesting a causal relationship between anxiety-like behavior and reduction in glutamate release in the ventral hippocampus of PRS rats (*Marrocco et al., 2012*). Moreover, antidepressant treatment such as agomelatine and fluoxetine corrected the glutamatergic release as well as anxiety-like behavior and social memory in PRS rats (*Marrocco et al., 2014*). The reduction of glutamate release and emotional behaviors are also corrected by the positive allosteric modulator of AMPA receptors reinforcing the central role of the glutamatergic system in PRS rats. Furthemore, GluRs are altered by early life stress. Indeed, PRS decreases the expression of mGlu2/3 receptors in the hippocampus of pups at postnatal day 22 (PND22) (*Laloux et al., 2012*) as well as in adult rats in both sexes (*Zuena et al., 2008*). mGluR2 and mGluR3 transcripts as well as mGluR2/3 protein levels were also reduced in the frontal cortex of adult male mice submitted to a prenatal stress (*Matrisciano et al., 2012*). In addition, PRS induces long-lasting alteration of mGluR5 protein levels

characterized by the decrease of mGluR5 in the hippocampus of pups at PND14 and PND22 (*Laloux et al., 2012*) as well as in aged rats (*Gatta et al., 2018*). Interestingly, in adult PRS rats, mGluR5 has been shown to be reduced in males but not in females (*Zuena et al., 2008*). These data suggest that the alteration of the glutamatergic synapse in brain region sensitive to stress, such as hippocampus and frontal cortex, lies at the core of the pathological phenotype caused by early life stress.

3. GABA

The interplay between synaptic excitation and inhibition lies at the core of brain function. An alteration of the balance between neuronal excitation and inhibition has been found in several neurological disorders such as epilepsy, schizophrenia, autism, stress-related disorders. GABA (*gamma*-aminobutyric acid) is the major inhibitory neurotransmitter in the mature mammalian central nervous system (*Bowery and Smart, 2006*).

3.A. GABA metabolism

Glutamate and GABA metabolism are intimately linked because glutaminase converts glutamine to glutamate. GABA is synthesized by decarboxylation of glutamate by glutamic acid decarboxylase (GAD) (*Roberts and Frankel, 1950*). GAD exists in two isoforms, GAD65and GAD67, which have different molecular weights (65 and 67KDa), catalytic and kinetic properties, and subcellular localization (*Walls et al., 2010*). GABA is stocked inside synaptic vesicles via vGAT (vesicular GABA transporter). The action potential triggers the increase of intracellular calcium and lead to the GABA release inside the synaptic cleft. GABA bind GABAergic receptors expressed in the post-synaptic membrane. GABA remaining in the synaptic cleft is reuptake by astrocyte mediated by GAT2 (GABA transporter type 2) or by the presynaptic neurons via GAT1. The GABA reuptake by glial cells and neurons is transformed to succinate and enter to Krebs (leading to ATP synthesis) to finally synthetize glutamate (in neurons) and glutamine (in astrocytes) that will be converted to GABA.

3.B. GABA receptors

GABA acts via two type of receptors: ionotropic $GABA_A$ receptor and metabotropic $GABA_B$ receptor.

$GABA_A$ receptors

In adult brain, the activation of $GABA_A$ receptors leads to fast hyperpolarization. The binding of GABA in $GABA_A$ receptors open the ion channel leading to the diffusion of chloride (Cl^-)

into the cell along its concentration gradient, thus hyperpolarizing the post-synaptic mature neuron (*Blednov et al 2014; Luján et al. 2005*). $GABA_A$ receptors are ligand-gated chloride ion channels comprised of α, β, γ and δ subunits in a heteropentameric structure (*Macdonald and Olsen 1994*; *Blednov et al. 2014*). More diversity exists within subunit families leading to further heterogeneity of the $GABA_A$ receptor (*Macdonald and Olsen 1994*). Depending of the subunit compositions, $GABA_A$ receptors are distributed differentially in the adult brain. Notably, receptors containing α1 and γ2 subunits localize in the synaptic cleft whereas receptors containing α4, α5, α6, and δ subunits localize extrasynaptically/perisynaptically. Extrasynaptic $GABA_A$ receptors are high-affinity GABA receptors implicated in tonic inhibition, whereas synaptic $GABA_A$ receptors are those involved in fast, phasic inhibition (*Farrant and Nusser, 2005*; *Lee and Maguire 2014*)

$GABA_B$ receptors

$GABA_B$ receptors ($GABA_BRs$) are the G protein-coupled receptors for the inhibitory neurotransmitter GABA. $GABA_BRs$ are composed of principal $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ subunits, which form the core of the receptor. Principal subunits form functional $GABA_{B(1a,2)}$ and $GABA_{B(1β,2)}$ heterodimers that shape higher-order oligomers (*Gassman and Bettler, 2012*). At the opposite to $GABA_A$ receptors, $GABA_B$ receptors are responsible for the later and slower component of inhibitory transmission (*Couve et al. 2000*). $GABA_BRs$ are localized at the pre and post synaptic membrane (*Misgeld et al. 1995*). Activation of these receptors mobilizes second messengers which then which then diffuse and activate various intracellular signal cascades and ultimately lead to either activation of postsynaptic K^+ channels or inhibition of presynaptic Ca2+ channels (*Couve et al. 2000*). At the presynaptic level, activation of GABAB receptors decrease Ca2+ conductance leading to a reduction of neurotransmitter release (*Misgeld et al. 1995*).

3.C. GABA: an excitatory neurotransmitter during development

While GABA is an inhibitory transmitter in the adult brain, GABA exerts excitatory effects in the developing brain. Early in development, including fetal stages and the first post-natal week in rodents, activation of $GABA_A$ receptors depolarizes the membrane and thus has excitatory effects (*Ben-Ari et al., 1989*; *Cherubini et al., 1991*). The GABAergic depolarization acts in synergy with NMDA receptor inducing depolarization and Ca^{2+} influx through voltage-operated Ca^{2+} channels (VOCC) that control wide spectrum of developmental phenomena including cell differentiation, migration, and synaptic plasticity (*Ben-Ari et al., 1989).* Until now, the change of GABA polarity has been shown to have two main phases: in

rats the first one happens shortly before delivery, where a rapid and completely reversed excitatory to inhibitory switch of GABA occurs. This event is triggered by maternal oxytocin in order to protect fetal neurons in increasing their resistance to insults during delivery (*Tyzio et al., 2006*). The second phase, usually assigned as "GABA switch", is a progressive and permanent switch starting soon after birth and is complete, in rodents, by the end of the first postnatal week (*Valeeva et al., 2013*). The molecular mechanism underpinned the GABA switch rely on the developmental induction of the expression of the neuronal (Cl^-)-extruding K^+/Cl^- co-transporter (KCC2) and the Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1). In immature neurons, NKCC1, a Cl^- importer, is highly expressed leading to an accumulation of Cl^- inside the cell. Because of this increased amount of this anion, $GABA_AR$ opening induces Cl^- efflux and hence generates membrane depolarization. At the contrary, mature neurons express higher levels of KCC2, a Cl^- exporter, resulting in a lower intracellular Cl^- concentration generating Cl- influx upon GABAAR activation and ultimately to the membrane hyperpolarization (figure12) (*Kakazu et al., 1999*; *Rivera et al., 1999*; *Dzhala et al., 2005*; *Ben-Ari et al., 2007*).

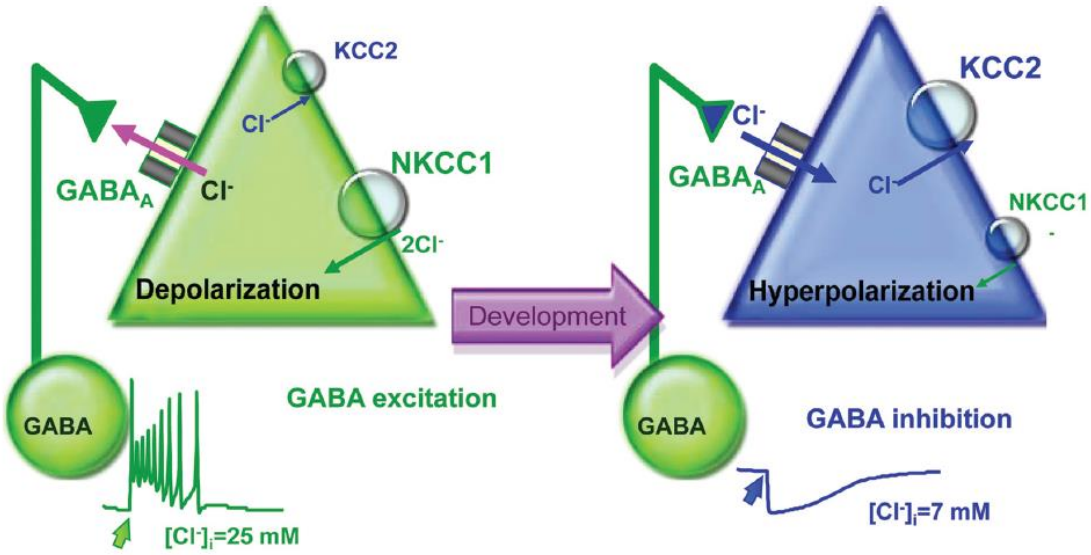

Figure 12. GABA shift. The GABA shift of actions is determined in part by a sequential development of two major chloride cotransporters, NKCC1 and KCC2. The former, which imports chloride, but not the latter, which exports it, is present in utero already. In more adult neurons, KCC2 fully operates, whereas NKCC1 is less active, leading to a higher accumulation of chloride in immature neurons (*From Ben-Ari et al., 2012*).

3.D. GABAergic interneurons

Although GABA interneurons (INs) are a minority population in the brain, they are essential for the control of inhibition (*Kepecs and Fishell, 2014*). The activity patterns of these

GABAergic interneurons (INs) play a critical role in information processing in cortex. To maximize flexibility, the cortex relies on the existence of a large diversity of GABAergic (*Ascoli et al., 2008*). This distinction of INs relies on their diversity in terms of morphology, connectivity, markers and physiological properties (figure 13). Cortical INs display a wide range of anatomical variety of somatic, dendritic and axonal morphologies. Moreover, INs's axons target specific subcellular domain (*Kubota, 2014*; *Markramet al., 2004*). INs exhibit different firing patterns, a consequence of the interplay of membrane cable properties and ion channel composition defining the passive and active membrane biophysical properties among IN subtype (*Kawaguchi and Kubota, 1997*; *Ascoli et al., 2008*). These properties impact IN responses to excitatory inputs and their postsynaptic impact onto target cells. Lastly, INs express different molecules such as calcium-binding proteins and neuropeptides (*Kepecs and Fishell, 2014*). All these characteristics put the light on a large diversity within the GABAergic interneuronal population, and particularly that they can have tremendous consequences on cellular and network neuronal circuits (*Tremblay et al., 2016*).

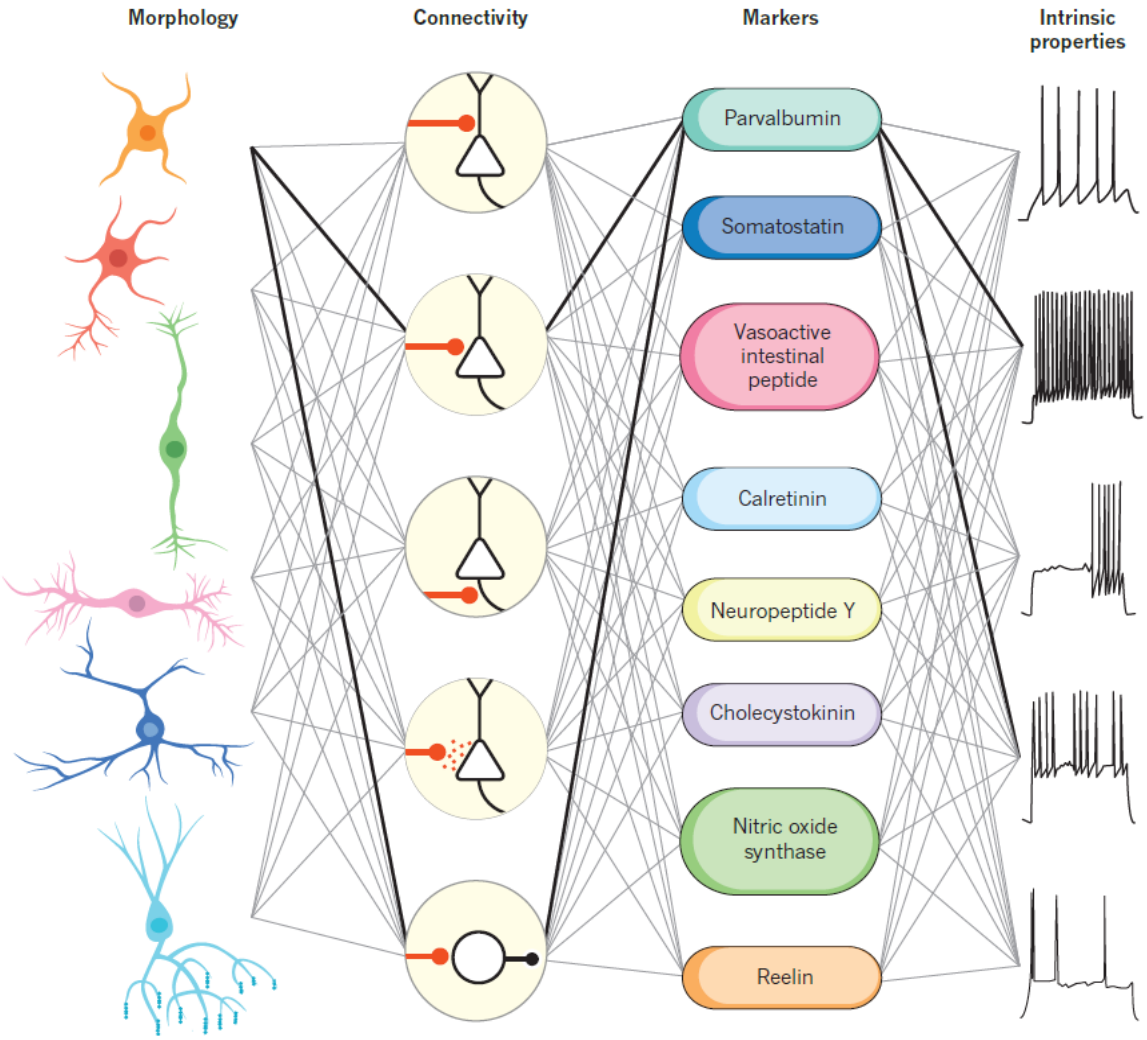

Figure 13 Multiple dimensions of interneuron diversity. Interneuron cell types are usually defined using a combination of criteria based on morphology, connectivity pattern, synaptic properties, marker expression and intrinsic firing properties. The highlighted connections define fast-spiking cortical basket cells (*from Kepecs et al., 2014*).

INs expressing the calcium-binding protein parvalbumin (PV) or the neuropeptide somatostatin (SST) represent 70% of cortical INs. Additionally, to these INs, the other 30% of INs express the ionotropic serotonin receptor 5HT3a. Importantly, these three markers are expressed in largely non-overlapping GABAergic interneurons populations in cortex (*Zeisel et al.,2015*; *Tasic et al., 2016*).

Parvalbumin interneurons

PV^+ interneurons (for interneurons expressing parvalbumin) exhibit fast spiking firing properties. Three different PV^+ interneurons has been found: chandelier cells, basket cells, and translaminar interneurons although translaminar interneurons constitute a relatively rare type of fast-spiking interneurons (*Bortone et al., 2014*). Chandelier cells have a very typical morphology because their shape is similar to a chandelier light fixture. Chandelier cells form synapses onto the axon initial segment of pyramidal cells (*Somogyi et al., 1982*). Chandelier cells are well represented between layers 1and 2 and in layer 6 (*Taniguchi et al., 2013*). On the other hand, PV^+ basket cells are the most abundant type of interneuron in the neocortex. Their axons form synapses on the soma and proximal dendrites of pyramidal cells and other interneurons (*Hu et al.,2014; Lim et al., 2018*).

Somatostatin interneurons

Interneurons expressing somatostatin (SST^+ interneurons) are a second class of interneurons that usually project to the dendrites. As for PV^+, SST^+ cells can be subdivided in two major interneurons: Martinotti cells and non-Martinotti cells. SST^+ Martinotti cells display ascending axon that arborizing profusely in layer 1 (*Xu et al., 2006; Hilscher et al., 2017*). They are particularly abundant in layer 5, but they can also be found easily in layers 2 and 3. Martinotti cells, often mediate disynaptic inhibition (*Silberberg and Markram, 2007*). Differentially, SST^+ non-Martinotti cells are abundant throughout layers 2 to 6 and particularly in layer 4 (*Ma et al., 2006*), where they primarily target PV+ basket cells (*Xu et al., 2013*). SST^+ non-Martinotti cells display lower resting membrane potential and higher firing frequency than Martinotti cells but lower than PV^+ basket cells (*Nigro et al., 2018*; *Lim et al., 2018*).

5HT3aR interneurons

The last and third class of cortical interneuron expresses the serotonin receptor 5HT3aR (*Rudy et al., 2011*).5HT3aR+ interneurons co-express mostly the vasoactive intestinal peptide (VIP). They principally target SST^+ and PV^+ interneurons (*Jiang et al., 2015*) thus displaying a disinhibitory effect. VIP^+ interneurons are bipolar cells with vertically oriented axons. They are abundant in layers 2 and 3, and often display continuous adapting firing properties (*Prönneke et al., 2015; Lim et al., 2018*) and co-express the calcium-binding protein calretinin (CR).

A very important question rising by the diversity observed in the GABAergic interneurons is to understand how such a diversity is created.

3.E. GABAergic interneurons development

Most GABAergic interneurons within the telencephalon arise from one of two embryonic subcortical progenitor zones, the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE). GABAergic interneurons are distributed through the amygdala, striatum, and cerebral cortex. Within the cortex, the MGE gives rise to the PV^+ and SST^+ interneurons. (Butt *et al., 2005; Fogarty et al., 2007).* The CGE produces relatively rarer subtypes, including neurogliaform, bipolar and vasointestinal peptide (VIP)-expressing multipolar interneurons (*Lee et al., 2010*). Importantly, genetic lineage analysis within the hippocampus reinforces the idea that specific interneurons arise from specific structures but demonstrates that a simple correspondence across forebrain regions is untenable. Accordingly, it has been shown in the cortex that neurogliaform neurons are CGE-derived but a large proportion of these neurogliaform neurons in the hippocampus arises from the MGE, (*Tricoire et al., 2010*) highlighting differences in GABAergic interneurons development across brain regions (*Kepecs et al., 2014*). In order to understand better the differentiation of GABAergic interneurons during development it has been necessary to map interneurons diversity onto molecular mechanism. All GABAergic neurons seem to use the transcription factor encoded by Dlx1, Dlx2, Ascl2, Gsx1 and Gsx2. Notably, Dlx1 and Dlx2 function at multiple stages of GABAergic maturation: in the acquisition of GABAergic identity (*Stühmer et al., 2002*), the initiation and cessation of tangential migration (*Cobos et al., 2007*) and in the morphological and physiological maturation of specific subclasses (*Cobos et al 2005).* Furthermore, Nkxx2-1 has emerged as a key regulator for the MGE-derived PV and SST lineages. Indeed, Nkxx2-1 promotes MGE-derived interneuron fates over CGE-derived cell types (*Sussel et al., 1999*).

There are currently two main hypotheses about the specification of GABAergic interneurons highlighted by Brie Wamsley and Gord Fishell. The first one suggests that interneuron identity is established approximately at birth through environmental cues that shape intrinsic progenitor identity. The most extreme version of this model posits that, upon generation, interneurons are bestowed with a covertly encoded blueprint that allows them to follow a precise differentiation and maturation program to develop into a specific subtype. The second possibility is that the genetic information necessary to shape the identity of interneuron subtypes is only

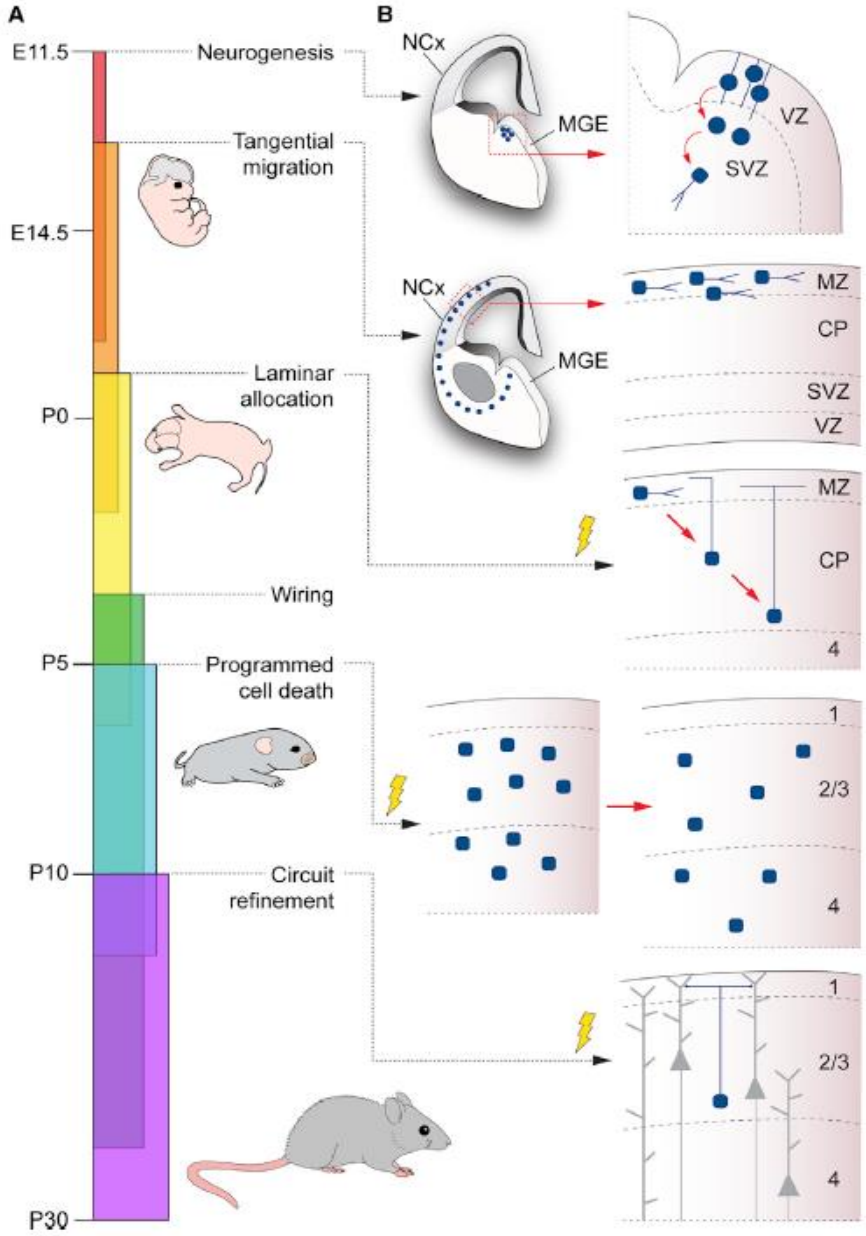

Figure 14. Milestones in the Development of Cortical Interneurons (A) Timeline of the development of cortical interneurons in the mouse. The main events have been highlighted in corresponding temporal periods: neurogenesis, tangential migration, laminar allocation (which involves radial migration), wiring (dendritic and axonal morphogenesis and establishment of synapses), programmed cell death, and circuit refinement. Interneuron identity is likely specified at neuronal birth, but it unfolds over a protracted period of time through which the final characteristics of each type of interneuron are acquired. (B) The development of layer 2/3 SST+ Martinotti cells is used here as an example to illustrate the main developmental milestones in the generation of cortical interneurons in mice. At least a population of SST+ Martinotti cells is generated from progenitor cells in the dorsal aspect of the MGE. SST+ Martinotti cells preferentially migrate to the embryonic cortex

through the marginal zone (MZ) stream. During radial migration into the cortical plate (CP), SST+ Martinotti cells leave their trailing neurite in the MZ, which will eventually develop into a characteristic axonal arborization in layer 1. By the end of the first postnatal week, about 30% of interneurons undergo program cell death, including SST+ Martinotti cells. This process depends on the integration of these cells into cortical circuits. The surviving SST+ Martinotti cells remodel their synaptic connections during the second and third week of postnatal development. For example, layer 2/3 SST+ Martinotti cells end up establishing preferential connections with the apical dendrites of pyramidal cells also located in layer 2/3. The yellow thunderbolt symbol indicates processes that strongly depend on neuronal activity. MGE, medial ganglionic eminence ; NCx, neocortex; SVZ, subventricular zone; VZ, ventricular zone (*from Lim et al., 2018*).

acquired post-mitotically, later in development. The progressive specification model, like the progenitor specification model, posits that interneurons are restricted into a general class at birth. However, in this progressive specification scenario, the determinants of cortical interneuron subtype identity are established relatively late through the interaction with their cortical environment (*Wamsley and Fishell, 2017).*

A growing body of evidence demonstrates that the development of cortical and hippocampal GABAergic interneurons is altered by early life stress (*Fine et al., 2014*).

3.F. Early life stress and GABAergic interneurons

Prenatal stress delays inhibitory neuron progenitor migration in the developing neocortex associated with changes in two transcription factors, dlx2 and nkx2.1 (*Stevens et al., 2013*) that are involved in cortical interneuron migration (*Lim et al., 2018*; *Kepecs et al., 2014*). A follow up of this study showed that prenatal stress alters parvalbumin neurons proportions in adult medial prefrontal cortex and hippocampus. Moreover, number of GABAergic cells were correlated with behavioral tasks such as a lack of social preference, an increase of anxiety-like behavior on the elevated plus maze, and reduced time in the center of an open field (*Lussier and Stevens, 2016*). Accordingly, to the idea that GABAergic cells play a key role in emotional behaviors, it has been shown recently that alteration of GABAergic interneurons in the ventral hippocampus by prenatal stress is associated with anxiety-like behavior. Furthermore, prenatal stress alters neuronal oscillations characterized by an increase of theta and gamma power in the ventral hippocampus. Interestingly, parvalbumin intensity was also reduced by prenatal stress and perineuronal nets (a specialized extracellular matrix important for the maturation, synaptic stabilization and plasticity of PV^+ neurons) were increased alongside OTX2, a critical transcriptional factor for GABAergic interneurons development

(*Murthy et al., 2019*). Although prenatal stress seems to alter GABAergic development, a causal link among PV^+ interneurons, PNNs, OTX2 and anxiety-like behavior remains to be determined. In another study, PNNs and density of PV^+ interneurons were reduced in the hippocampus of heterozygous GAD67-GFP (Knock-In; $GAD67^{+/GFP}$) mice subjected to prenatal stress from embryonic day 15.0 to 17.5. Very interestingly, no change was observed in naïve mice and wild-type mice suggesting that both prenatal stress and reduced Gad1 gene expression are prerequisites for these changes (*Wang et al., 2018*; *Uchida et al., 2014*). Another study using the animal model of prenatal restraint stress in mice showed a decrease of protein levels of glutamic acid decarboxylase 67 (GAD67) and reelin protein level that was associated with an increase of MeCP2 bound to GAD67 promoter (*Matrisciano et al., 2012*). Notably other epigenetic linked to GABAergic interneurons has been found. Indeed, DNMT1 and DNMT3a were upregulated preferentially in GABAergic neurons of frontal cortex and hippocampus. Moreover, PRS increased binding of DNMT1 and MeCP2, and an increase in 5-methylcytosine and 5-hydroxymethylcytosine in specific CpG-rich regions of the reelin and GAD67 (*Matrisciano et al., 2013*). Maternal separation delays the timing of the GABA switch in the hippocampus and inhibited the increase in membrane KCC2 expression (*Furukawa et al., 2017*) and alters protein levels of GABA transporter (vGAT) and glutamic acid decarboxylase (GAD65) (*Martisova et al., 2012*). In addition to the increase of KCC2 induced by maternal separation, Hu and colleagues observed an increase of NKCC1. Interestingly, daily administration of bumetamide (a NKCC1 blocker) during the first two postnatal weeks decreased the expression of NKCC1, KCC2 in the hippocampus of stressed mice (*Hu et al., 2017*).

Taken together, these data strengthen the idea that early life stress impacts the developmental trajectory of GABAergic interneurons and have long-lasting effect of the GABAergic system. Investigating the links between early life stress and inhibitory systems of the forebrain is critic to enhance our understanding of the etiology and physiopathology for several psychiatric disorders in which inhibitory neurons pathology is implicated.

AIM OF THE THESIS

Nature and nurture (e.g genes and environment) shape, together, the behavior (*Meaney 2004*; *Normile, 2016*). Nature can be modeled by nurture through epigenetic process (*Meaney 2010*; *Powledg, 2011; Maccari et al., 2014; Maccari et al., 2017*). Adverse environment during perinatal period can lead to maladaptative responses resulting in an increase of the risk to develop diseases such as metabolic diseases (*Barker et al., 1995*) or psychiatric disorders involving the postnatal human relationship (*Maccari et al., 2017*). Hence, animal models of early life stress allow us to study in depth and understand better the consequences of early life events on the organism and how they shape neurobehavioral adaptations to environmental challenges later in life.

The perinatal stress rat model (PRS) is characterized by abnormalities of synaptic transmission in the hippocampus, which enhances the vulnerability to stress-related phenotypes in the adult rat life (*Marrocco et al., 2012*; *Marrocco et al., 2014*; *Mairesse et al., 2015*; *Morley-Fletcher et al., 2018*). The decrease of the risk-taking behavior observed in males PRS is associated with a selective reduction of glutamate release. By using antidepressants or drugs cocktail that increase the glutamate release, the altered phenotype triggered by early life stress was reversed (*Marrocco et al. 2012*; *Marrocco et al., 2014*) suggesting that glutamatergic synapse impairment lies at the core of the altered phenotype caused by early life stress. Accordingly, mGlu2/3 receptors, regulating the glutamate release, are reduced in males PRS during early life as well as in both sexes in adult PRS (*Zuena et al., 2008*). Differentially to mGluR2/3, PRS reduces the expression of mGluR5 in hippocampus in males but not in females (*Zuena et al., 2008*). Sexual differences are also observed at a behavioral level where males PRS display decrease of risk-taking behavior (measured in the elevated plus maze) but not females, which increase risk-taking behavior (*Zuena et al., 2008*). Moreover, PRS differentially affects addictive behaviors in males and females (*Reynaert et al., 2016*). Gonadal hormones that contribute to sex differences in stress responsivity are modified by early life stress (*Goel and Bale, 2008*). Plasma dihydrotestosterone (DHT) levels are increased in adult male PRS and female PRS rats show lower plasma estradiol (E_2) levels. Interestingly, this estradiol decrease was still observed in middle-aged female rats highlighting that PRS accelerates the ageing-related-disruption in the estrous cycle (*Van Camp et al., 2018*). PRS affects also other important aging-related process as depicted by the

increase of pro-inflammatory markers (*Vanbesien-Mailliot et al., 2007*), the inhibition of neurogenesis in the hippocampus (*Lemaire et al., 2000*) and the accelerating aging of the HPA axis (*Maccari et al., 1995*). Consistently, cognitive deficits are observed in aged male rats as showed by the impairment of the spatial memory in Y-maze (*Valle et al., 1999*) strengthen the idea that PRS could have long-lasting effects on aging-related process notably involving the hippocampus. With the hippocampus, the alteration of the development of the nigrostriatal system represents also risk factor for age-related disorders. Several evidences suggest that PRS modulate the nigrostriatal system. Indeed, PRS affects the symmetry of D_2 receptor expression in the medial caudate/putamen (*Adrover et al., 2007*), and to restrain haloperidol-induced catalepsy and enhance apomorphine-induced stereotypies (*Marrocco et al., 2013*) in adult rats. Although the effects of PRS on the dopaminergic system in the nucleus accumbens are well established, very little is known about how PRS programs the dopaminergic synapse in the nigrostriatal system. Some evidences suggest that glutamatergic projections to the striatum could play an important role by modulating the dopaminergic system (*Lipska et al., 1995*). Besides its close connection with the dopaminergic system, glutamate interacts very closely with the GABAergic system.

The excitatory-inhibitory balance is essential in shaping neuronal networks. Disruption of this balance is associated with neuropsychiatric disorders, such as schizophrenia and autism (*Yoon et al., 2010*; *Hashimoto et al., 2003*; *Horder et al., 2018*). This excitatory-inhibitory balance is critically regulated by mGlu2 and mGlu3 receptors mostly because they modulate glutamate release from presynaptic terminals. The group I metabotropic glutamate receptors are also known to be important because they regulate a variety of ion channels, leading to the alteration of neuronal excitability (*El-Hassar et al. 2011*; *Yu et al., 2018*). Other studies have linked metabotropic glutamatergic receptors to GABAergic system. Pharmacological activation of mGlu group II and I reduce the amplitude of GABA-mediated inhibitory postsynaptic currents in the CA1 region of the hippocampus (*Liu et al., 1993*; *Jouvenceau et al., 1995*). These data suggest that mGlu2 and mGlu3 could be involved in the development of the GABAergic system.

Within this context, the objective of my thesis was to shed new light on **the role of early life stress and metabotropic glutamate receptors on the central nervous system development across life span**.

In **chapter 1**, we investigated the long-lasting effects of PRS on the dopaminergic synapse in basal ganglia and on glutamate system in rats. To this aim, we used the perinatal restraint

stress (PRS) in rats, an animal model well characterized and used for almost three decades by Pr. Maccari's team.

a) Since sex differences are poorly studied and because PRS alters aging-related process, we first investigated long-term effects induced by PRS in aged male and female rats with a focus on behaviors and on the glutamatergic synapse across different brain regions involved in the stress-response.

b) Because alteration of the nigrostriatal system development represents a risk factor for age-related disorders, we analyzed the long-lasting effects of perinatal stress on neurochemical, electrophysiological, and behavioral correlates of striatal motor function in adult and old male rats.

The alteration of the glutamatergic synapse is core in the phenotype triggered by PRS (*Marrocco et al., 2012*; *Marrocco et al., 2014*, *Morley-Fletcher et al., 2018*) as discussed in the chapter one, which strengthens considerably this idea. One of the most striking results was the decrease of mGlu2/3 receptors in ventral hippocampus in aged males and females PRS rats. Because mGlu2/3 receptors are also reduced by PRS in pups (*Laloux et al., 2012*) and adult rats (*Zuena et al., 2008*) we wanted further investigate the role of mGlu2 and mGlu3 receptors in the CNS development. Several evidences suggest that mGlu2 and mGlu3 interact with GABAergic system as demonstrated for example by epigenetic modifications on reelin and GAD67 promoters induced by activation of mGlu2/3 receptors (*Matrisciano et al., 2011*). Moreover, the deletion of mGlu3 receptors in mice impairs working memory (*Lainiola et al., 2014*; *Fujioka et al., 2014*), a cognitive process linked to GABAergic system in prefrontal cortex (*Enomoto et al., 2011*; *Banuelos and Wołoszynowska-Fraser 2017*). Furthermore, GABA plays a key role in brain development because it shapes and refines the neuronal circuits during early brain development (*Lim et al, 2018*).

Hence, the **chapter 2** was dedicated to elucidate the role of mGlu2 and mGlu3 receptors in the developmental trajectory of cortical GABAergic interneurons. Using knockout mice for mGlu2 and mGlu3 receptors we analyzed critical events that shapes the developmental trajectory of GABAergic interneurons during early-postnatal days.

In the **chapter 3**, we moved to the investigation of the interaction between genes and environment that shape brain development and behavior. Hence, we studied the interplay between mGlu3 receptors and early life stress. To this aim, we used pregnant wild-type mice and mGlu3$^{-/-}$ mice that underwent to a maternal restraint stress (MRS) during a sensitive time window for GABAergic interneurons proliferation and migration. Following this procedure,

we analyzed several GABAergic interneurons related genes as well as stress and epigenetic markers in the hippocampus during the first 30 days post-natal using real-time PCR

RESULTS

CHAPTER 1

LONG-LASTING EFFECTS OF PRS ON GLUTAMATERGIC SYNAPSE AND BASAL GANGLIA

There is a growing interest on how stressful events occurring early in life influence brain development shaping the risk to develop CNS disorders in the adult life. So far, most of the studies have focused on the programming effects of early life stress in adult males. For example, rats exposed to a perinatal stress (PRS) display a decrease of the risk-taking behavior, social impairment in the adult life. This phenotype triggered by PRS is associated with a hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, a reduced glutamatergic transmission in the ventral hippocampus, and changes in the expression of mGlu2/3 and mGlu5 receptors (*Zuena et al., 2008*; *Marrocco et al., 2012*; *Marrocco et al., 2014*). Importantly, PRS differentially affects males and females at a behavioral and neurochemical level (*Zuena et al., 2008*; *Reynaert et al., 2014*). In the previous years, it is becoming clear that considering the sex as a biological variable is essential for our understanding of factors contributing to disease vulnerability or resilience. Growing body of evidences demonstrated that sex differences across lifespan differ significantly between males and females, from development until aging. These differences are notably driven by the role of gonadal hormones and disparities in gene dosage and regulatory mechanisms (*Ward, 1972*; *Bale, 2019*; *Nugent et al., 2018*; *Lee et al., 2017*; *Bale 2016*; *Bronson 2017*; *McCarthy and Arnold, 2011*).

Although, the programming effects triggered by early life stress are becoming clearer, very little is known about the long-lasting effects of PRS in both sexes.

Hence, the first part of the chapter one aimed to elucidate the life-long program triggered by PRS in aged male and female rats. In other words, we investigated how early life stress impacts aging process in male and female rats.

In this context, we carried out a behavioral characterization of aged male and female PRS rats. Then, we performed proteomic analysis in different brain region involved in stress response

with a focus on the glutamatergic synapse, synaptic vesicle-associated proteins as well as proteins involved in the stress response.

In the **second part of the chapter one**, we moved from the evidence that PRS has a profound impact on aged male rats to investigate the effects of PRS on the basal ganglia system associated with motor behavior. Aging is associated with motor impairment and alteration of the nigro-striatal system (*Volkow et al., 1998*; *Alder et al., 2002*). The dopaminergic system plays a central role in the nigro-striatal system related to motor function (*Kalia and Land, 2015*). Early life stress influences the normal expression of dopaminergic receptors and impairs dopaminergic neurotransmission and metabolism (*Diaz et al., 1995*, *1997*; *Fride and Weinstock, 1989*). Moreover, rodents submitted to perinatal stress display an alteration of the symmetry of D2 receptor expression in the medial caudate/putamen (*Adrover et al., 2007*), and to restrain haloperidol-induced catalepsy and enhance apomorphine-induced stereotypies (*Marrocco et al., 2013*). Only few studies focused on the impact of early life stress on the nigro-striatal system and the dopaminergic system in adult life and no studies have been ever performed in old animals. Taken together, these data drove us to examine nigro-striatal programming induced by perinatal stress in a longitudinal study in adult and old male rats. To this aim, we used neurochemical, electrophysiological and behavioral correlates of striatal motor programming, and examined if the changed observed in adult were persistent in aged rats.

All together these articles show that maternal stress programs dysmasculinization of the glutamatergic synapse in aged offspring suggesting that males are more sensitive than females to PRS. Hence, PRS induced long-term effects on basal ganglia motor system involving nigrostriatal dopaminergic system indicating an accelerated aging.

ARTICLE 1

MATERNAL STRESS PROGRAMS DYSMASCULINIZATION OF THE GLUTAMATERGIC SYNAPSE IN AGED OFFSPRING

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Abstract

Studies on programming by early life stress in animal models have been essentially limited to adult males. Using the perinatal stress (PRS) rat model we investigated the programming effects in aged male and female rats. We show that, at the behavioral level, PRS decreased risk-taking behavior, spatial memory, and gross and fine motor skills specifically in males. In contrast, female PRS showed an increased risk-taking behavior but no change in spatial memory and gross or fine motor skills. A common effect induced by PRS in both sexes was the diminution of the exploratory behavior. Impressively, we observed a massive hypofunction of the glutamatergic synapse (receptors and synaptic vesicles-associated proteins) in the hippocampus (ventral and dorsal) and in prefrontal cortex only in males. PRS decreased glucocorticoid (GR) and mineralocorticoid (MR) receptors in males whereas it increased MR receptors, GFAP and BDNF in females. Multidimensional analysis revealed that PRS induces a dysmasculinization profile of the glutamatergic synapse in hippocampus, ventral and dorsal, and prefrontal cortex while it induces a dysmasculinization profile of stress markers only in the dorsal hippocampus. The reduction of testosterone and the increased of estradiol levels in plasma and aromatase expression support the dysmasculinization triggered by PRS. This could explain, in part, the higher vulnerability displayed by males against PRS effects during aging while increased MR, BDNF expression and GFAP in female rats may act as protective factors towards PRS and aging effects.

Keywords (4-6): early restraint stress, aging, MR/GR, sex hormones, long-term programming, dysmasculinization

1. Introduction

Aging is characterized by a progressive loss of physiological integrity, leading to impaired function and increased risk of death (Carlos Lopez-Otin et al., 2013). Stress is well known to contribute to the variability of the aging process and to the development of age-related neuropsychopathologies (Heim and Nemeroff, 1999; McEwen, 2002; Miller and O'Callaghan, 2005). The glucocorticoid stress hormones regulate a cohort of physiological functions such as intermediary metabolism and the immune system and influence development, growth and aging (Gray et al., 2017). Excess of glucocorticoids as occurs during chronic stress may alter the physiological aging process (Sapolsky et al., 1986 Lupien et al., 2009; McEwen et al., 2020). Studies in animals and human have shown that stressful events occurring during critical period of brain development have lifelong altered programming effects (de Rooij et al., 2010; Maccari et al., 2017). Early life stress impacts cognitive processing during aging as demonstrated in middle-aged and aged rodents performing the Y-maze test and touch panel operant task (Vallee et al., 1999; Yajima et al., 2018). Perinatal stress (PRS) affects other aging-related processes in adult male rats as demonstrated by the increase of pro-inflammatory markers (Vanbesien-Mailliot et al., 2007), the inhibition of neurogenesis in the hippocampus (Lemaire et al., 2000) and the accelerating aging of the HPA axis (Maccari et al., 1995, Vallée et al., 1999). Furthermore, glutamate (Nicoletti et al., 2011), the most abundant excitatory neurotransmitter in the CNS, is deeply involved in stress-related disorders (Popoli et al., 2011; Kadriu et al., 2019). Glutamatergic pyramidal neurons that furnish corticocortical connections between the association cortices as well as excitatory hippocampal connections are particularly vulnerable to aging (Morrison and Baxter, 2012; Morrison and Hof, 1997). Importantly, glutamate plays a key role in programming effects induced by PRS. Indeed, PRS massively reduces glutamate release in the ventral hippocampus of adult male rats, which is associated with a reduced expression of synaptic vesicle-associated proteins (Marrocco et al., 2012; Mairesse et al., 2015; Morley-Fletcher et al., 2018). Enhancing the glutamate release using a cocktail of mGlu2/3 and $GABA_B$ receptor antagonists reversed the alterations in risk-taking behavior in PRS rats (Marrocco et al., 2012), reinforcing the idea that the impairment of the glutamatergic synapse in the ventral hippocampus lies at the core of the pathological phenotype triggered by PRS.

In women and men, the decline in gonadal hormones occurring during aging, in addition to cellular aging processes are associated with stress deregulation (McEwen 2002; Bale and Epperson, 2015). Furthermore, gonadal hormones are altered by PRS as demonstrated by

increased plasma dihydrotestosterone (DHT) levels in adult male PRS rats and lower plasma estradiol (E_2) levels in adult females PRS (Reynaert et al., 2016). Interestingly, the decrease of estradiol levels was still observed in middle-aged female rats highlighting that PRS accelerates the aging-related-disruption in the estrous cycle (Van-Camp et al., 2018). These hormonal changes are associated with sex differences in behavior in adult PRS rats. For example, PRS decreases risk-taking behavior (measured in the elevated plus maze) in males but not in females (Zuena et al., 2008). Moreover, PRS differentially affects addictive behaviors in males and females (Reynaert et al., 2016). Other studies using prenatal stress paradigms have previously reported disruptions of sex differences in behavior, morphology, sex hormones and gene expression profiles (Ward, 1972; Meisel et al., 1979; Reznikov et al., 1999; Kapoor and Matthews, 2005; Biala et al., 2010). This highlight that early perinatal period of life represents a specific window of sensitivity during which offspring is susceptible to the programming effects of PRS. Most of the studies concerning perinatal stressful events have focused in adult males. To our knowledge, no study has ever analyzed the effects of PRS during aging in males and females. In order to fill this gap, we investigated the long-term programming effects of PRS in both sexes on behaviors, glutamatergic synapses, stress and neuroplasticity markers, which are known to be sex dependent. To this purpose, we used the PRS rat model, in which exposure of the pregnant mother to restraint stress reduces maternal behavior, to study neuroplasticity in brain regions sensitive to stress (ventral and dorsal hippocampus, prefrontal cortex and striatum), and cognitive and motor behaviors in 21-22 months aged male and female rats.

2. MATERIAL AND METHODS

Experimental design

The experimental timeline is shown in **Figure 1**. After the PRS procedure consisting of the-restraint stress of pregnant mothers and in the reduction of maternal behavior in the first postpartum week, behavioral and biochemical measurements in the hippocampus (ventral and dorsal) and the prefrontal cortex in both males and females (21-22 months) have been studied. Behavioral tests were performed when rats were 21 months old. One week after the last behavioral test brain structures and blood (plasma and serum) were collected. All experiments followed the rules of European Communities Council Directive 86/609/EEC. The Local Committee CEEA-75 (Comité d'Ethique en Experimentation Animale Nord-Pas de Calais, 75) approved the experimental procedures.

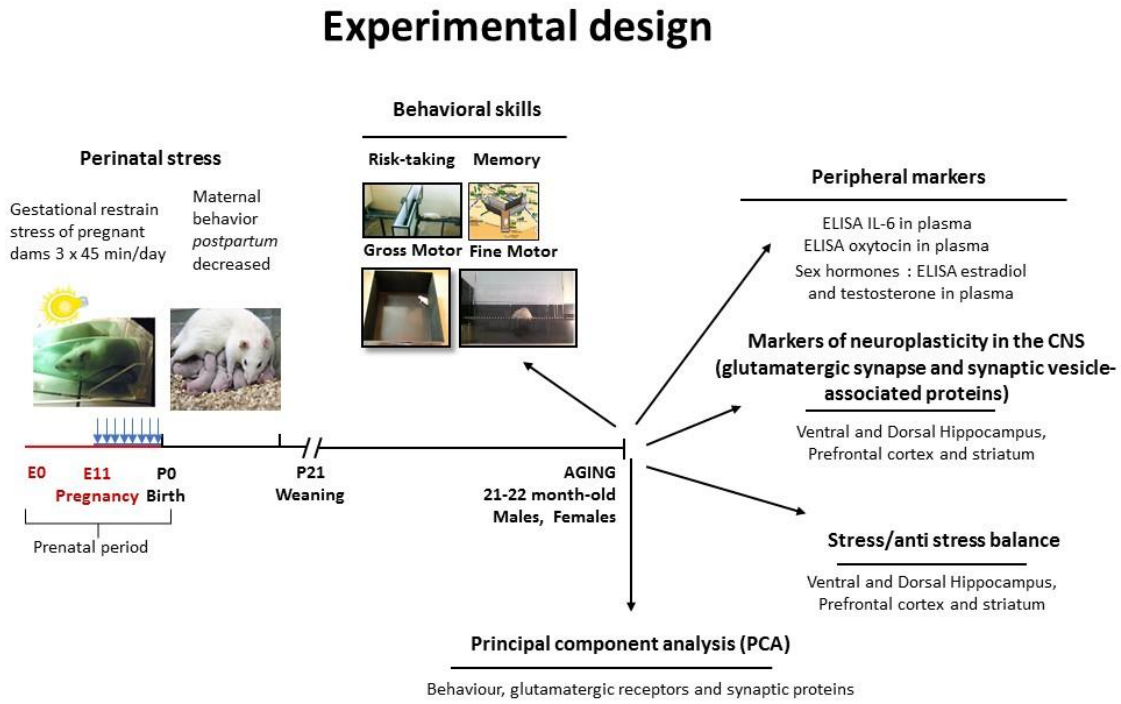

Fig 1 Experimental design and timeline
Induction of PRS and analysis of maternal behavior in the first *postpartum* week were followed by behavioral, and biochemical measurements in the old (21-22 months old) male and female progeny as indicated. Several multidimensional analysis have been performed.

2.1 Animals

Thirty nulliparous female Sprague–Dawley rats, weighing approximately 250 g, were purchased from Charles River (France) and housed under standard conditions with a 12 h light/dark cycle. After group housing (5 females/cage) for two weeks, each female was individually housed for one week with a

sexually experienced male rat. Following that, a gain of at least 10 grams was considered as index of pregnant status.

2.2 Stress procedure

The stress procedure was performed on 1 set of breeding, using 30 females (15 CONT and 15 stressed dams). PRS, i.e., the adult offspring of dams exposed to multiple episodes of restraint stress during pregnancy causing reduced maternal care, was obtained according to our standard protocol (Maccari et al., 1995) and showed in the **Fig. 1**. Briefly, from day 11 of pregnancy until delivery, pregnant females were subjected to restraint in a transparent plastic cylinder and exposed to bright light during three daily sessions of 45 minutes. Control pregnant females were left undisturbed in their home cages and were handled once per week during body weight gain. After weaning, male and female offspring from litter with a balanced sex ratio were used for the experiments. Animals were housed in-group of two or three and maintained under similar environmental condition during their entire life span, 21-22 months-old rats were used in all experiments.

2.3 Behavioral studies

2.3.1 Risk taking behavior in elevated-plus maze test and exploratory behavior

Behavior of PRS or control progeny was assessed in the elevated-plus maze (EPM) (Pellow et al., 1985; Morley-Fletcher et al., 2018). Briefly, the test was performed early in the afternoon between 1.00 p.m. and 4.00 p.m., lasted for 5 min and began with the placement of the rat in the center of the maze with the head facing a closed arm. We used a custom-made EPM apparatus as described by Vallée et al. 1999, with closed and open arms 20 x 20 cm. The luminosity of closed arms was about 25 lux and the luminosity of open arms was about 50 lux. Behavior was recorded on-line by a video camera and manually scored by a trained observer blind to animals' conditions (PRS and control) by using a specific software (Noldus, The Observer®). The time spent in open and closed arms was measured and the percentage of time spent in open arms was calculated and analyzed as risk-taking behavior. The number of closed arms entries was analyzed as exploratory behavior.

2.3.2 Fine motor skills in ladder-rung walking test

The horizontal ladder rung walking test apparatus (Metz and Whishaw, 2002) consisted of side walls made of clear plexiglas and metal rungs (3 mm diameter), which could be inserted to create a floor with a minimum distance of 1 cm between rungs. The sidewalls were 1 m long and 19 cm high measured from the height of the rungs. The ladder was elevated 30 cm above the ground with a refuge

(home cage) at the end. Varying the position of the metal rungs modified the difficulty of the task. A regular pattern of the rungs allowed the animals to learn the pattern over several training sessions and to anticipate the position of the rungs. After the training sessions an irregular pattern has been made in order to analyze how rats managed to cross the ladder with an increase of the difficulty. The test was recording using two video cameras. One video camera was placed in front of the first half of the ladder in a slight ventral angle and the other was placed in front of the second half of the ladder in a slight ventral angle in order to analyze precisely the misplacement of the rats' paw. Following the video recording the foot faults were scored. A score of one was attributed when the rat's paw was misplaced and a score of two was attributed when the rat's paw severely slipped or missed. Then, the percent increase in errors between the last training and the test with the irregular pattern was calculated as follow: (error score irregular pattern-error score last training)/ error score last training *100.

2.3.3 Spatial recognition memory in Y maze and exploratory behavior

Spatial recognition memory was measured in a two-trial memory task in a Y maze (Vallee et al., 1999) made of grey plastic with three identical arms (50 cm) enclosed with 32-cm high sidewall and illuminated by dim light (40 lux). Each arm was equipped with two infrared beams, one at each end of the arm. The floor of the maze was covered with rat odor-saturated sawdust, and between each session the sawdust was mixed in order to eliminate olfactory cues. Visual cues were placed in the testing room and kept constant during the behavioral testing sessions. The task consisted of two trials separated by a time interval. In the first trial (acquisition phase), one arm of the Y maze was closed, and animals could visit the two other arms for 5 min. During the inter-trial interval (ITI), rats were housed in their home cages located in a room different from the test room. During the second trial (retention phase), animals had free access to the three arms and were again allowed to explore the maze for 5 min. The time spent in the novel arm (previously closed in the first trial) was calculated as a percentage of the total time spent in all three arms during the first 3 min of the second trial; time spent in the novel arm above chance (i.e. 33%) indicates spatial recognition. Memory performance was tested with an ITI =6 h. Total arm entries for 5 minutes was also analyzed as a measure of exploratory behavior.

2.3.4 Gross motor skills-locomotor activity in the open field arena

Exploratory behavior was evaluated by placing a rat into a corner of an open-field arena (100x100x50 cm) and allowing the rat to freely explore the field for 10min. Lightning was approximatively 60lux inside the arena. Activity and the trajectory length in the open field was recorded and quantified by Video Track® (Viewpoint, Lyon France).

2.4 Western blot analysis

Hippocampus (ventral and dorsal), striatum and prefrontal cortex of control (CONT) and PRS, male and female rats were rapidly dissected and immediately stored at -80°C. Glutamatergic proteins and synaptic vesicle-associated proteins were assessed in synaptosomes. To isolate synaptosomes, tissue was manually homogenized with a potter in 10 volumes of HEPES-buffered sucrose (0.32M sucrose, 4mM HEPES pH 7.4). All procedures were performed at 4°C. Homogenates were centrifuged at 1000 g for 10 min, and the resulting supernatants were centrifuged at 10,000 g for 15 min. The pellet obtained from the second centrifugation was re-suspended in 10 vol of HEPES-buffered sucrose (Marrocco et al., 2012). This pellet contained the crude synaptosomal fraction. BCA assay were used to determine protein concentration. Synaptosome lysates were re-suspended in Laemmli reducing buffer and 15µg for synaptosomal fraction or 30µg from total homogenates of each sample were loaded. Two gels were needed to carry all samples; one intern control was loaded in the different gels in order to compare samples from different gels together.

Proteins from synaptosomal fraction and total homogenate were first separated by electrophoresis on SDS-polyacrylamide gels according to their molecular weight and then transferred to nitrocellulose membranes (Bio-Rad). Transfer was performed at 4°C in a buffer containing 35mM Tris, 192mM glycine and 20% methanol. After transfer, blots were incubated in a blocking solution containing Tris-buffered saline (TBS), 5% (w/v) non-fat milk. We used the following primary antibodies on synaptosomal fraction: mouse polyclonal anti-SNAP25, rabbit polyclonal anti-synapsin Ia/b (1:4000; catalog #20780), rabbit polyclonal anti-synaptophysin (1:8000; catalog #9116), rabbit polyclonal anti-syntaxin (1:4000, catalog #13994), rabbit polyclonal anti-synapsin IIa (1:4000; catalog #25538), all purchased from Santa Cruz Biotechnology; mouse monoclonal anti-rab3a (1:2000; catalog #107111), mouse monoclonal anti-Munc-18 (1:2000; catalog #116011), mouse polyclonal anti-VAMP (1:1500; catalog #104 111) which were purchased from Synaptic Systems; rabbit polyclonal anti-mGlu5 receptors (1:1000; catalog #AB5675), rabbit polyclonal anti-mGlu2/3 receptors (1:1000; catalog #06-676) all purchased from Millipore; rabbit polyclonal anti-GluN1 (1:2000; catalog #EPR2481(2)) purchased from Abcam, GluA2, GluA3, GluN2a, GluN2b, xCT, vGlut1, vGlut2. We used the following primary antibodies on total homogenates: GR, MR, OXTR, GFAP, BDNF and aromatase. To ensure that each lane was loaded with an equivalent amount of proteins, the blots were probed with a mouse monoclonal anti-β-actin (1:5000; catalog #A5316, Sigma). All primary antibodies were incubated overnight at 4°C. HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (purchased from GE-Healthcare) were used at a dilution of 1:7500 and were incubated for 1 h at room temperature. Bands were visualized with an enhanced chemiluminescence system (ECL enhancer Thermofisher). After immunoblotting, digitized images of bands immunoreactive for target antibodies

and actin were acquired (FUSION®) and the area of immunoreactivity corresponding to each band was measured using the ImageJ imaging software. A ratio of target to actin was then determined, and these values were compared for statistical significance.

2.5 Measurement of interleukin-6 levels, oxytocin and sex hormones

Interleukin-6 (pg/ml), oxytocin (pg/ml), testosterone (ng/ml) and estradiol levels (pg/ml) were determined in plasma extracted from blood samples.

Plasma was collected using EDTA as an anticoagulant and centrifuged for 15 minutes at 1000 g at 4°C. Plasma was stored at -20°C until assessment. All ELISA Kits were used according to the manufacturer's protocol. All standards, blood samples, and controls were analyzed concurrently, in duplicate. The optical density (OD) of the sample was determined at 450 nm using a microplate reader (BioTek Instruments, Winooski, USA).

ELISA kit	Manufacture	Sensibility range
Interleukin-6	CUSABIO (CSB-E04640r)	0.312 pg/ml-20 pg/ml
Oxytocin	CUSABIO (CSB-E14197r)	7.5 pg/ml-600 pg/ml
Testosterone	DEMEDITECH (DEV9911)	0.066 pg/ml-25 pg/ml
Estradiol	DEMEDITECH (DEV9999)	2.5 pg/ml-1.280 pg/ml

2.6 Multidimensional analyses

To see how sex and stress affects protein quantities or behavior in a more general manner, we performed multidimensional analysis on sets of multiple proteins or behaviors. Proteins were categorized as glutamatergic signaling proteins or as synaptic vesicle proteins, and analyses were carried out for each protein set in each brain region.

Since protein levels tended to be correlated with each other, we used principal components analysis (PCA) to summarize the variation in a protein set and for plotting. For PCA, individuals who were missing more than 50% of protein measurements were excluded. Missing values were replaced with the mean of the Sex-by-PRS group. To quantify distances between groups, we used the mean of the Manhattan distances between each individual in one group with the centroid of another group. We created a "dysmasculinization score", meant to represent the phenomenon in which PRS causes males to become more similar to females in various aspects. The dysmasculinization score was the distance from control males to control females divided by the distance from PRS males to control females. A score greater than 1 thus signifies that PRS males are more similar to control females than control males are.

2.7 Partial correlation

To determine significant relationships between two measures, we calculated the correlation between the two measures after controlling for Sex and Stress, using MANOVA on the following linear model, $Y = \beta_0 + \beta_1*X + \beta_2*Sex + \beta_3*Stress + \beta_4*Sex*Stress + \varepsilon$. Generally, Y is a behavior and X is a neurophysiological measure such as protein or hormone levels. Significant $\beta_1 \neq 0$ indicates significant correlation between X and Y.

2.8 Statistical analysis

Behavioral and biochemical data were expressed as the mean ± SEM and analyzed using a parametric analysis of variance (ANOVA) with group (CONT *vs.* PRS) and sex (males and females) as independent variables. When group x sex interaction was present, post-hoc comparisons were performed with Fisher test. The level of significance was set at $p<0.05$.

Permutational MANOVA (PERMANOVA) (Anderson, 2017) was used to test group differences for principal component analysis (PCA). A Mann-Whitney-Wilcoxon test was used to determine if the difference between these distances were significant.

RESULTS

Effect of sex and PRS on behaviors and peripheral markers during aging

We carried out a behavioral characterization of aged male and female PRS rats. *Risk-taking behavior* was assessed in the elevated plus maze (EPM) (**Fig 2A**) analyzing both latency to open arm and time spent in open arm. We observed a sex-dimorphic profile that was inverted by PRS (latency to open arm: *Group x Sex effect*, $F_{(1,35)}=19.184$, p=0.0001; % time spent in the open arm, $F_{(1,35)}=13.821$, p=0.0007; n=8-12 rats/group). We found a clear-cut sex effect in the EPM for unstressed control rats, with increased latency to enter the open arm in females with respect to males (Post-hoc Fisher, p=0.014). Remarkably, PRS females showed reduced latency (Fisher, p=0.00088) and increased time spent in open arm (Fisher, p=0.00006) with respect to PRS males. Furthermore, PRS in females decreased the latency (Fisher, p=0.0056) and increased the time spent in open arm as compared to controls (Fisher, p=0.003). PRS in males increased latency (Fisher, p=0.0025) and reduced time spent in open arm compare to controls (Fisher, p=0.02). Thus, PRS increased risk-taking behavior in females while reduced it in males. *Spatial recognition memory* was studied in the Y-maze and is represented by the recognition score as shown in **figure 2B**. The recognition score was changed (recognition score (%): *Group x Sex effect*, $F_{(1,29)}= 4.641$, p=0.04; n=8-9 rats/group) driven by the diminution of the time spent in the novel arm in PRS males compared to control male rats (Post-hoc Fisher, p=0.014) and

relative to PRS females (Fisher, p=0.04). Overall, PRS did not affect spatial learning in females but it decreased it in males. In order to study fine motor skills, we submitted the animals to the *ladder rung walking test* (**Fig. 2C**) analyzing the number of errors. PRS increased the percentage of errors in both sexes *(Group effect*: $F_{(1,35)}$=5.235, p=0.028, n=8-12 rats/group). Furthermore, males displayed higher percentage of errors than females (*Sex effect*: $F_{(1,35)}$=4.55, p=0.04, n=8-12 rats/group). The overall reduction of percentage of errors by PRS was mainly driven by males (Fisher, p=0.012) than females (Fisher, p=0.92). The *exploratory behavior* was assessed in three different tests (**Fig 2D**). In the EPM, PRS reduced the closed arm entries in both sexes (closed arm entries*: Group effect*: $F_{(1,35)}$=5.147, p=0.04, n=8-12 rats/group). In the open field, PRS reduced the distance travelled in both sexes (distance travelled (cm): *Group effect*, $F_{(1,36)}$=11.004, p=0.002; n=8-11 rats/group). Furthermore, females showed higher distance travelled than males (distance travelled (cm): *Sex effect*, $F_{(1,36)}$=20.823, p=0.00005; n=8-11 rats/group). The same profile was observed in the Y-maze where PRS decreased the total arms entries (total arms entries: *Group effect*, $F_{(1,29)}$=11.023, p=0.0024; n=8-9 rats/group) and, moreover, females showed increased total arm entries compare to males (total arms entries: *Sex effect*, $F_{(1,29)}$=4.251, p=<0.048; n=8-9 rats/group). Hence, PRS reduced the exploratory behavior in both sexes in all three tests. *Peripheral markers* were measured and are shown in **figure 2E**. No change in plasmatic oxytocin level was observed (n=4-6 rats/group), while for IL-6 levels we noticed a group x sex interaction (IL-6: *Group x Sex effect*, $F_{(1,20)}$=22.901, p=0.00007; n=5-9 rats/group). PRS led to a massive decrease of IL-6 in females (Fisher, p=0.00031), as opposed to male PRS, which displayed higher IL-6 levels than male controls (Fisher, p=0.024). Furthermore, female controls showed an increase of IL-6 compared to male controls (Fisher, p=0.000004). In addition, *partial correlations* have been performed to determine significant relationships between behaviors and the two peripheral markers after controlling for sex and PRS group. We found a negative and positive correlation between risk-taking behavior in the EPM respectively for IL6 ((Beh.|(Sex, Stress) p=0.013; n= (5-9 rats/group; **Fig. 2F**)) and plasma oxytocin levels ((Beh.|(Sex, Stress) p=0.027; n=4-5 rats/group; **Fig 2G**)).

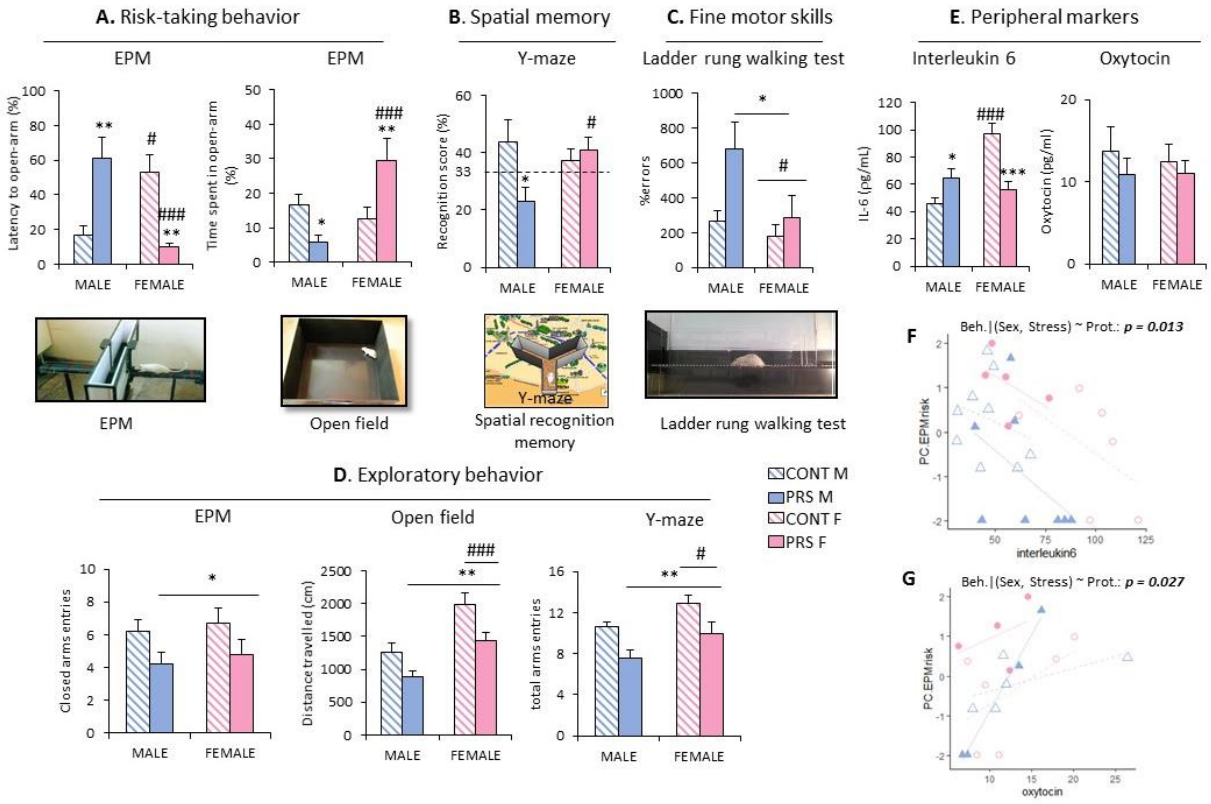

Fig 2 Effect of sex and PRS on behaviors and peripheral markers during aging
Risk-taking behavior in the elevated plus maze is shown in **2A.** The latency to open arm and the time spent in open arm were the two parameters analyzed. The spatial recognition memory was study using the Y-maze. The recognition score (%) is represented in **2B**. The ladder rung-walking test was used to study fine motor skills. The % of errors is shown in **2C.** The exploratory behavior was analyzed in the elevated plus maze considering the closed arms entries, in the open field with the distance travelled (n=8-11 rats/group) as well as in the Y-maze where we analyzed the total arm entries (**2D**). Oxytocin (pg/m) and interleukin-6 (pg/m) levels in plasma are shown in **2E**. Partial correlation between risk-taking behavior and IL-6 is represented in **2F**. Partial correlation between risk-taking behavior and oxytocin levels is shown in **2G**. Error bars represent SEM. CONT *vs* PRS * = p<0.05; **= p<0.01; ***= p<0.001. Males vs Females # = p<0.05; ##= p<0.01; ###= p<0.001.

Effect of sex and PRS on neuroplasticity of glutamatergic synapses in aged rats

We analyzed by western blot, proteins related to the glutamatergic synapse, including metabotropic and ionotropic glutamate receptors and glutamate transporters in brain regions sensitive to stress (**Fig. 3**). In the *ventral hippocampus* (**Fig. 3A**) PRS decreased *mGlu2/3 receptors* in both sexes (*Group effect*, $F_{(1,17)}$=8.472, p=0.0097; n=5-6 rats/group). Moreover, females, independently of the group, showed a diminution of mGlu2/3 receptor protein levels compared to males (*Sex effect*, $F_{(1,17)}$=4.827, p=0.042). *mGlu5 receptors* (*Group x Sex effect*, $F_{(1,23)}$=3.734, p=0.063; n=6-7 rats/group) were reduced in male PRS rats (Fisher, p=0.049) and female controls (Fisher, p=0.034) with respect to control males. In addition, females displayed a reduction in *post-synaptic density protein 95* (PSD95) (*Sex effect*, $F_{(1,19)}$=6.919, p=0.016; n=5-7 rats/group). *The GluN1* subunit of NMDA receptors (*Group per sex effect*, $F_{(1,23)}$=7.362, p=0.012, n=6-7 rats/group) was reduced in males PRS (Fisher, p=0.0013) and

in CONT females (Fisher, p=0.00018) compared to control males. Differentially to GluN1, *GluN2A* protein levels were increased in females with respect to males (*Sex effect*, $F_{(1,17)}$=6.944, p=0.017; n=2-8 rats/group). Interestingly, female PRS rats showed an opposite profile for vGlut1 and vGlut2. Indeed, *vGlut1* (n=3-4 rats/group) was decreased in PRS females (Fisher, p=0.04) while *vGlut2* expression (n=5-8 rats/group) was higher than PRS males (Fisher, p=0.028). *xCT* protein levels were modified in the ventral hippocampus (*Group x sex effect*, $F_{(1,22)}$=12.394, p=0.0019, n=5-7 rats/group). In particular, *xCT protein* levels increased in male PRS rats (Fisher, p=0.0026) and in control females (Fisher, p=0.00008) compared to male control rats. A selection of immunoblots of **Fig. 3A** are shown in supplementary **Fig. S1**. To examine how sex and PRS affect protein levels in a more general manner, we performed *multidimensional analysis* on sets of protein related to glutamatergic synapse (**Fig. 3B**). This revealed that PRS induces a dysmasculinization of the glutamatergic synapse in ventral hippocampus (*dysmasculinization score*: 1.41; Mann-Whitney-Wilcoxon test, p=0.005; n=5-7 rats/group).

In the *dorsal hippocampus* (**Fig. 3C**) no difference in *mGlu2/3* protein levels were observed. Similarly to what found in the ventral hippocampus, *mGlu5 receptors* in the dorsal hippocampus were modified (*Group x sex effect*, $F_{(1,21)}$=13.886, p=0.0012, n=5-7 rats/group). We observed largely reduction of mGlu5 receptors in male PRS rats (Fisher, p=0.00004) and females control (Fisher, p=0.0011) with respect to control males. *GluN1 protein levels* were reduced in females (*Sex effect*, $F_{(1,23)}$=10.308; n=6-7 rats/group). *The GluA2* subunit of AMPA receptors was reduced by PRS in both sexes (*Group effect*, $F_{(1,22)}$=6.179, p=0.021; n=5-9 rats/group). We noticed a group x sex interaction for *GluN2b* (*Group x sex effect*, $F_{(1,21)}$=11.696, p=0.0026, n=4-9 rats/group), *GluA3* (*Group x sex effect*, $F_{(1,22)}$=4.964, p=0.036, n=5-9 rats/group), *vGlut1* (*Group x sex effect*, $F_{(1,21)}$=10.885, p=0.0034, n=4-9 rats/group) and *xCT* (*Group x sex effect*, $F_{(1,21)}$=16.044, p=0.00064, n=5-7 rats/group). Post-hoc analysis revealed that PRS males displayed reductions in protein levels with respect to control males for *GluN2b* (Fisher, p=0.00023), *GluA3* (Fisher, p=0.009), *vGlut1* (Fisher, p=0.00024) and *xCT* (Fisher, p=0.00084). Similarly, control females showed a reduction of *GluN2B* (Fisher, p=0.00022), *GluA3* (Fisher, p=0.037), *vGlut1* (Fisher, p=0.00082) and *xCT* (Fisher, p=0.014) with respect to control males. A selection of immunoblots of **Fig. 3C** are shown in supplementary **Fig. S2**. Similarly to what found in the ventral hippocampus, there was an overall dysmasculinization profile of the glutamatergic synapse induced by PRS in the dorsal hippocampus (**Fig. 3D**) (*dysmasculinization score*: 1.61; Mann-Whitney-Wilcoxon test, p=0.0009; n=5-9 rats/group).

Interestingly, we observed a reduction of *mGlu2/3* receptor protein levels in response to PRS in the *prefrontal cortex* (**Fig. 3E**; *Group effect*, $F_{(1,22)}$=13.858, p=0.0011 n=6-7 rats/group) and in female rats (*Sex effect*, $F_{(1,22)}$= 7.436, p=0.012 n=6-7 rats/group). Also, *the mGlu5 receptors* were modified (*Group x sex effect*, $F_{(1,22)}$=3.093, p=0.093, n=6-7 rats/group). In particular, we observed a reduction in PRS males (Fisher, p=0.02) and control females (Fisher, p=0.035) compared to CONT males.

Moreover, protein levels of *PSD95* were decreased by PRS (*Group effect*, $F_{(1,22)}$=25.134, p=0.00005; n=6-7 rats/group) and in female rats (*Sex effect*, $F_{(1,22)}$=5.47, p=0.029; n=6-7 rats/group). PRS also reduced *GluN1* protein levels in both sexes (*Group effect*, $F_{(1,22)}$=5.615, p=0.027; n=6-7 rats/group). A Group x Sex interaction was found for *GluN2B* ($F_{(1,18)}$=6.658, p=0.019, n=4-7 rats/group), *GluA2* ($F_{(1,20)}$=3.494, p=0.076; n=6-7 rats/group) and *GluA3* ($F_{(1,22)}$=11.528, p=0.0026; n=5-7 rats/group). In particular, PRS males displayed a reduction of protein expression respectively to control males for GluN2B (Fisher, p=0.0045; p=0.00096), GluA2 (Fisher, p=0.016) and GluA3 (Fisher, p=0.0001). Similarly, control females showed reduced protein levels of GluN2b (Fisher, p=0.0004), GluA2 (Fisher, p=0.0059) and GluA3 (Fisher, p=0.0004) compared to control males. Furthermore, females of both groups showed an increase of xCT protein levels (*Sex effect*, $F_{(1,19)}$=5.76, p=0.027; n=4-7 rats/group). A selection of immunoblots of **Fig. 3E** are shown in supplementary **Fig. S3**. In prefrontal cortex, we again see a pattern of dysmasculinization profile of the glutamatergic synapse triggered by PRS (**Fig. 3F**) (*dysmasculinization score*: 1.91; Mann-Whitney-Wilcoxon test, p=0.0003; n=5-7 rats/group).

In the *striatum* (**Fig. 3G**), protein levels of *PSD95* (*Group x sex effect*, $F_{(1,18)}$=11.978, p=0.0028; n=4-7 rats/group) were reduced in PRS males (Fisher, p=0.0013) and control females (p=0.00029) compared to control males. Moreover, PRS increased *GluN1* (*Group x sex effect*, $F_{(1,23)}$=5.24, p=0.032, n=6-7 rats/group) only in females (Fisher, p=0.0056). *GluA2* was decreased in females of both groups (*Sex effect*: $F_{(1,18)}$=4.872, p=0.04; n=4-6 rats/group). Concerning *vGlut2* protein levels, we observed a sex-dimorphic profile that was inverted by PRS (*Group x sex effect*, $F_{(3,18)}$=9.119, p=0.0074; n=4-7 rats/group). PRS decreased vGlut2 in males (Fisher, p=0.035) but increased vGlut2 in females (Fisher, p=0.0036) with respect to control males. A selection of immunoblots of **Fig. 3G** is shown in supplementary **Fig. S4**. Contrary to hippocampus (ventral and dorsal) and prefrontal cortex, there was not a dysmasculinization profile of the glutamatergic synapse in the striatum (**Fig. 3H**) (*dysmasculinization score*: 1.38; Mann-Whitney-Wilcoxon test, p=1; n=5-7 rats/group).

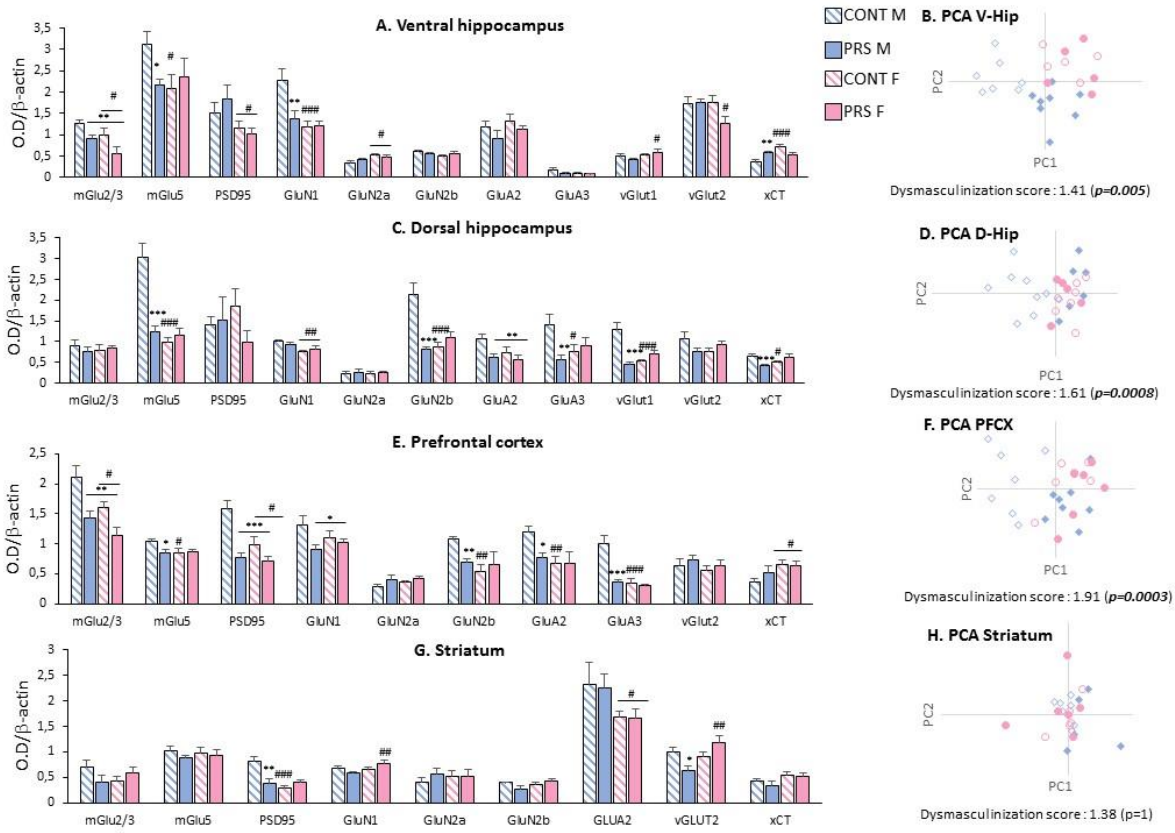

Fig 3 Effect of sex and PRS on neuroplasticity of glutamatergic synapses in aged rats
Immunoblot analysis of metabotropic, ionotropic glutamate receptors and glutamate transporters in synaptosomal fractions collected from the ventral hippocampus (**A**), dorsal hippocampus (**C**), prefrontal cortex (**E**) and striatum (**G**) of aged male and female PRS and control (CONT) rats. Muldimensional analysis of glutamatergic proteins in ventral hippocampus (**B**), dorsal hippocampus (**D**), prefrontal cortex (**F**) and striatum (**H**) are represented. Error bars represent SEM. CONT *vs* PRS * = p<0.05; **= p<0.01; ***= p<0.001. Males vs Females # = p<0.05; ##= p<0.01; ###= p<0.001.

Effect of sex and PRS on synaptic vesicles-associated proteins levels in aged rats

In the *ventral hippocampus* (**Fig. 4A**) we observed a *Group x Sex interaction* for protein levels of *SNAP25* ($F_{(1,23)}$=6.879, p=0.015, n=6-7 rats/group), *Syntaxin* ($F_{(1,23)}$=14.301, p=0.00096, n=6-7 rats/group) and *Rab3a* ($F_{(1,23)}$=5.981, p=0,022, n=6-7 rats/group). Indeed, PRS decreased the expression of SNAP25 (Fisher, p=0.0051), Syntaxin (Fisher, p=0.00014), and Rab3a (Fisher, p=0.0018) specifically in males. Moreover, we noticed a decrease of SNAP25 (Fisher, p<0.00007), Syntaxin (Fisher, p=0.00012) and Rab3a (Fisher, p=0.0034) in control females with respect to control males. A selection of immunoblots of **Fig. 4A** is shown in supplementary **Fig. S5**. By considering all these results together, multidimensional analysis revealed that PRS induced a dysmasculinization profile of synaptic vesicle-associated proteins (**Fig. 4B**) (*dysmasculinization score*: 1.58; Mann-Whitney-Wilcoxon test, p=0.0057; n=6-7 rats/group).

Fewer changes were found in the *dorsal hippocampus* (**Fig. 4C**). PRS reduced *SNAP25* in both sexes (*Group effect*, $F_{(1,23)}$=6.487, p=0.018; n=6-7 rats/group). In addition, SNAP25 (*sex effect*, $F_{(1,23)}$=5.484, p=0.02) and *SYP* (*sex effect*, $F_{(1,23)}$=4.956, p=0.036; n=6-7 rats/group) were decreased in females of both groups. A selection of immunoblots of **Fig. 4C** is shown in supplementary **Fig. S6**. Although we did not observed huge change of protein levels in the dorsal hippocampus, we observed a dysmasculinization profile of synaptic proteins induced by PRS (**Fig. 4D**) (*dysmasculinization score*: 1.66; Mann-Whitney-Wilcoxon test, p=0.026; n=6-7 rats/group).

In the prefrontal cortex (**Fig. 4E**), *syntaxin* expression was modified (*Group x sex effect*, $F_{(1,23)}$=14.301, p=0.067, n=6-7 rats/group) and was only reduced in PRS males compared to control males (Fisher, p=0.015). In contrast, *synapsinIIa* was increased by PRS in both sexes (*Group effect*, $F_{(1,23)}$= 5.318, p=0.011; n=6-7 rats/group). A selection of immunoblots of **Fig. 4E** is shown in supplementary **Fig. S7**. Considering all protein data, *a multidimensional analysis* showed that PRS led to a dysmasculinization of synaptic vesicle-associated protein profile in the prefrontal cortex (**Fig. 4F)** (*dysmasculinization score*: 1.29; Mann-Whitney-Wilcoxon test, p=0.049; n=6-7 rats/group).

In the striatum, (**Fig. 4G**), a *Group x Sex interaction* was showed for the expression of *SNAP25* ($F_{(1,23)}$=8.441, p=0.008; n=6-7 rats/group), *Munc-18* ($F_{(1,23)}$=4.26, p=0.05; n=5-7 rats/group) and *VAMP* ($F_{(1,21)}$=11.51, p=0.0027; n=5-7 rats/group). Indeed, PRS males displayed a reduction of SNAP25 (Fisher, p=0.0022), Munc-18 (Fisher, p=0.017) and VAMP (Fisher, p=0.014) compared to control males. Moreover, female controls showed reduced protein levels of SNAP25 (Fisher, p=0.019). Similarly, protein levels of *synapsinIab* were modified (*Group x sex effect*, $F_{(1,24)}$=11.51, p=0.074; n=7 rats/group) and were decreased in control females compared to control males (p=0.02). A selection of immunoblots of **Fig. 4G** is shown in supplementary **Fig. S8**. As opposed to what found in the ventral hippocampus, *dorsal hippocampus and prefrontal cortex*, no dysmasculinization profile of synaptic vesicle-associated proteins was observed in the striatum with *multidimensional analysis* (**Fig. 4H**) (*dysmasculinization score*: 0.99; Mann-Whitney-Wilcoxon test, p=0.61; n=6-7 rats/group). This result was similar to that found with glutamatergic proteins.

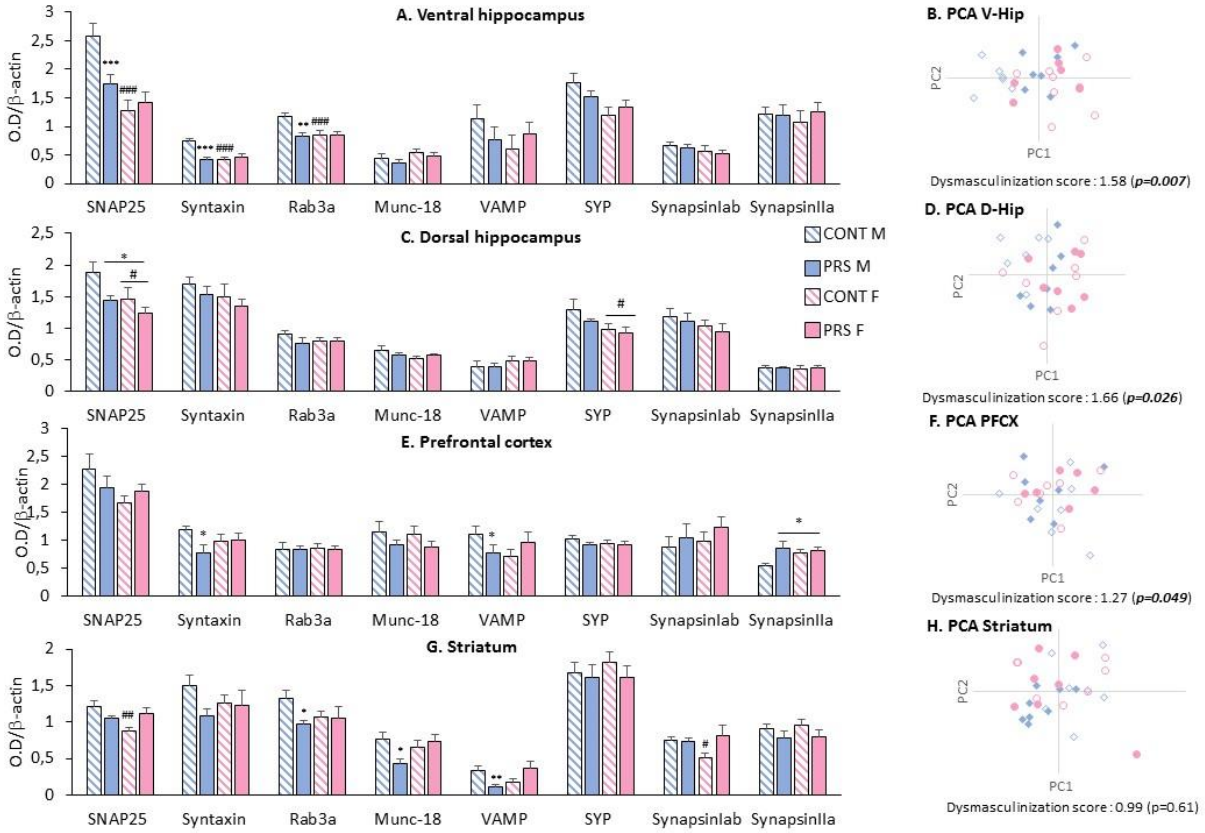

Fig. 4 Effect of sex and PRS on the expression of synaptic vesicle-associated proteins in aged rats
Immunoblot analysis of SNAREs and vesicle-associated proteins in synaptosomal fractions collected from the ventral hippocampus (**A**), dorsal hippocampus (**C**), prefrontal cortex (**E**) and striatum (**G**) of aged male and female PRS and control (CONT) rats. Principal component analysis of synaptic vesicle associated proteins in ventral hippocampus (**B**), dorsal hippocampus (**D**), prefrontal cortex (**F**) and striatum (**H**) are represented. Error bars represent SEM. CONT *vs* PRS * = p<0.05; **= p<0.01; ***= p<0.001. Males vs Females # = p<0.05; ##= p<0.01; ###= p<0.001.

Effect of sex and PRS on receptors for stress and anti-stress hormones and BDNF protein levels in aged rats

In the *ventral hippocampus* (**Fig. 5A**), *GR protein levels* are modified (*Group x sex effect*, $F_{(1,23)}$=2.913, p=0.101, n=6-7 rats/group) and were reduced by PRS only in males (Fisher, p=0.014). The same profile was observed for *MR* (*Group x sex effect*, $F_{(1,22)}$=7.703, p=0.011, n=5-7 rats/group) where PRS males showed a reduction of MR as compared to control males (p=0.02). Interestingly, PRS females showed higher protein expression of GR (Fisher, p=0.025) and MR (Fisher, p=0.031) than PRS males. Moreover, also protein levels of *OXTR* are modified (*Group x sex effect*, $F_{(1,23)}$=8.325, p=0.0083, n=6-7 rats/group) and were higher in control females than control males (Fisher, p=0.007) but PRS reduced OXTR expression in females (Fisher, p=0.02). Interestingly, *BDNF* was increased in females, independently of the group (*sex effect*, $F_{(1,22)}$=6.354, p=0.019; n=6-7 rats/group). PRS did not induced dysmasculinization of the neuroplasticity markers in the ventral hippocampus (**Fig. 5B**) (*dysmasculinization score*: 1.21; Mann-Whitney-Wilcoxon test, p=0.19; n=6-7 rats/group).

In dorsal hippocampus (**Fig. 5C)**, *MR protein levels* were altered (*Group x sex effect*, $F_{(1,23)}$=10.853, p=0.0032, n=6-7 rats/group) and were higher in PRS males (Fisher, p=0.0082) and control females (Fisher, p=0.0011) compared to control males. A sex-dimorphic profile was observed for *GFAP* (*Group x sex effect*, $F_{(1,22)}$=8.690, p=0.0074, n=5-7 rats/group). Indeed, PRS reduced the expression of GFAP only in males (Fisher, p=0.008) meanwhile GFAP was increased in PRS females respectively to males (Fisher, p=0.033). A multidimensional analysis revealed a dysmasculinization profile of the neuroplasticity markers induced by PRS in the dorsal hippocampus (**Fig. 5D**) (*dysmasculinization score*: 1.42; Mann-Whitney-Wilcoxon test, p=0.0021; n=6-7 rats/group).

In the prefrontal cortex (**Fig. 5E**), *MRs* were modified (*Group x sex effect*, $F_{(1,20)}$ =6.486, p=0.019, n=6 rats/group) and were increased in PRS females with respect to PRS males (Fisher, p=0.036) and compared to control females (Fisher, p=0.028). Furthermore, females in both groups displayed higher protein expression of *OXTR* (*Sex effect*, $F_{(1,19)}$=8.829, p=0.01; n=5-6 rats/group). Interestingly, we found a higher expression of *BDNF* (*Group effect*, $F_{(1,20)}$=13.705, p=0.0014; n=6 rats/group) and GFAP (*Group effect*, GFAP, $F_{(1,19)}$=16.466, p=0.00067; n=6 rats/group) in females of both groups. A selection of immunoblots of **Fig. 5E** is shown in supplementary **Fig. S11**. We did not observe a dysmasculinization of the neuroplasticity markers induced by PRS in the prefrontal cortex (**Fig. 5F**) (*dysmasculinization score*: 1.57; Mann-Whitney-Wilcoxon test, p=0.066; n=5-7 rats/group).

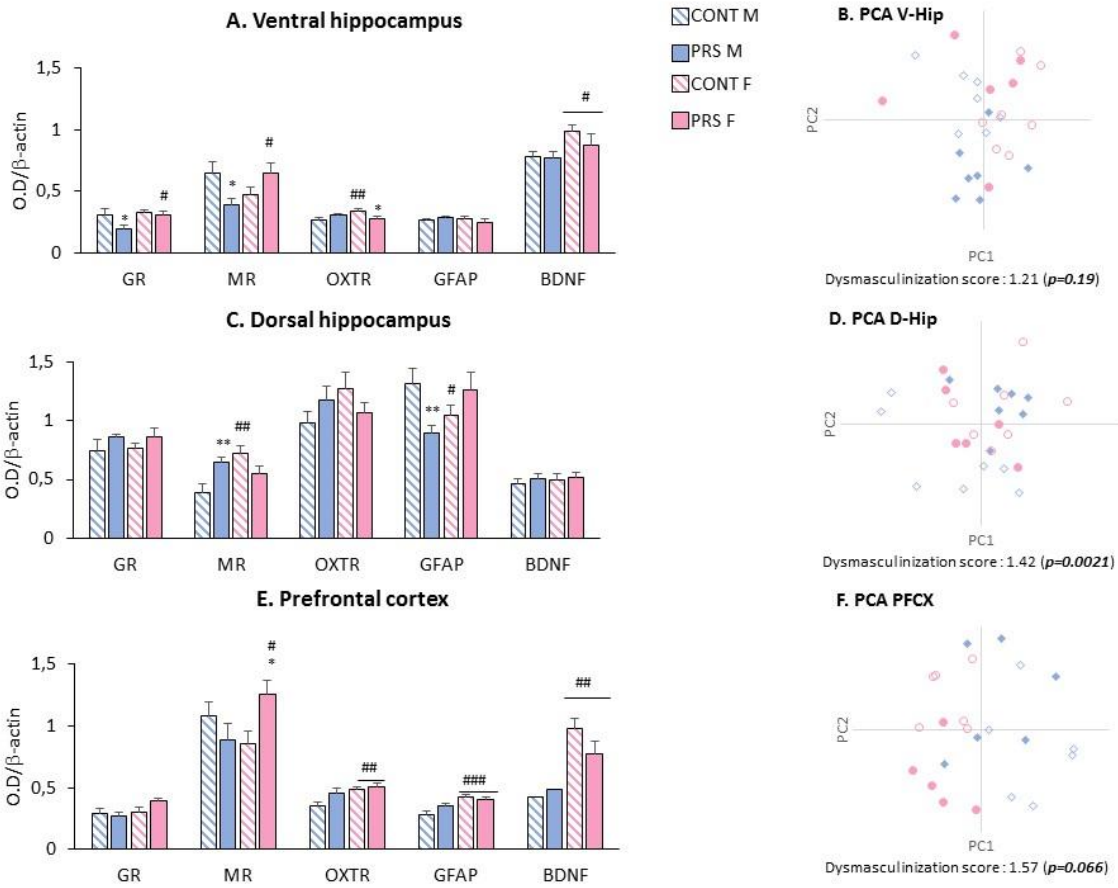

Fig. 5 Effect of sex and PRS on neurobiological markers of neuroplasticity in aged rats
Immunoblot analysis of GR, MR, OXTR, GFAP and BDNF in total homogenates collected from the ventral hippocampus (**A**), dorsal hippocampus (**C**) and prefrontal cortex (**E**) of aged male and female PRS and control (CONT) rats. Principal component analysis of neurobiological markers of neuroplasticity in ventral hippocampus (**B**), dorsal hippocampus (**D**), prefrontal cortex (**F**) are shown. Error bars represent SEM. CONT *vs* PRS * = p<0.05; **= p<0.01; ***= p<0.001. Males vs Females # = p<0.05; ##= p<0.01; ###= p<0.001.

Effect of sex and PRS on sex hormones in aged rats

Because sex hormones are known to be one of the main factors leading to sex differences, we assessed plasma testosterone and estradiol levels (**Fig. 6**). We found a significant reduction of *testosterone* by PRS in both sexes (*Group effect*, $F_{(1,24)}$=5.882, p=0.023; n=9-5 rats/group. Moreover, females displayed lower levels of testosterone than males (*Sex effect*, $F_{(1,24)}$=4.42, p=0.046; n=7-4 rats/group) (**Fig. 6A**). *Estradiol levels* in plasma revealed a *Group x Sex interaction* ($F_{(1,\ 21)}$=5.3, p=0.032; n=6 rats/group) where PRS females showed lower levels of estradiol than PRS males (Fisher, p=0.034) and PRS males a trend to a increase of estradiol levels (Fisher, p=0.2) (**Fig. 6B**). Protein levels of *aromatase*, the enzyme that converts testosterone to estradiol, were assessed by western blot analysis in the dorsal hippocampus (**Fig. 6C**). We observed a sex-dimorphic profile induced by PRS (*Group x sex effect*, $F_{(1,23)}$=12.071, p=0.0021; n=6-7 rats/group), with increased aromatase levels in PRS males (Fisher, p=0.0062) and reduced levels displayed by PRS females with respect to PRS males.

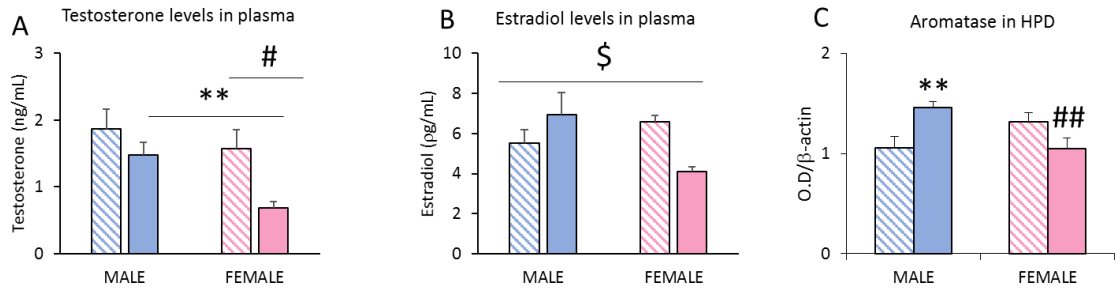

Fig.6 Effect of sex and PRS on sex hormones in aged rats
Testosterone (ng/m) estradiol (pg/mL) levels in plasma are respectively shown in **A** and **B**. Immunoblot analysis of aromatase in total homogenates collected from the dorsal hippocampus of aged male and female PRS and control (CONT) rat is represented in **C**.
Error bars represent SEM. CONT *vs* PRS * = p<0.05; **= p<0.01; ***= p<0.001. Males vs Females # = p<0.05; ##= p<0.01; ###= p<0.001; $: group x sex interaction.

DISCUSSION

We showed that perinatal stress cause life-long changes in mechanisms that shape the balance between vulnerability and resilience to stress, and, remarkably, the direction of changes were sex-dependent. At a behavioral level, PRS increased risk-taking behavior in females but decreased it in males. These results are reminiscent of those observed in adult rats (Zuena et

al., 2008) indicating that the sex-dependent programming induced by PRS is persistent and uniform. While spatial memory was impaired by PRS in males, no changes were observed in females. These results are also consistent with previous findings obtained in males (Vallee et al., 1999) and suggest that females are resilience to changes in neuroplasticity caused by early life stress. Motor performance declines during aging as shown by an increased variability of movement (Contreras-Vidal et al., 1998; Darling et al., 1989), slowing of movement (Diggles-Buckles, 1993), coordination difficulty (Seidler et al., 2002) and difficulties with balance and gait (Tang & Woollacott, 1996). Using the ladder rung walking test, we showed that PRS increased the numbers of errors essentially in males, lending credit to the hypothesis that aged females cope better with PRS even if PRS decreased the exploratory behavior in elevated-plus maze, Y maze and open field equally in both sexes. The immune system is altered by PRS during adulthood as demonstrated by a pro-inflammatory state (Vanbesien-Mailliot et al., 2007). Here, we have reported that PRS increases IL-6 levels in males but decreased IL-6 levels in females. It is known that IL-6 levels increase with age in both rats (Foster et al., 1992) and humans (Fagiolo et al., 1993). Thus, our findings suggest that females are protected against age-dependent inflammation caused by early life stress. Interestingly, rats with higher levels of IL-6 showed a reduced risk-taking behavior in the EPM, and this negative correlation is consistent with the current belief that inflammation highly contributes to behavioral changes associated with age and age-related disorders. Finally, levels of the anti-stress hormone oxytocin, which corrects behavioral and biochemical abnormalities caused PRS in adult rats (Gatta et al., 2018), showed a positive correlation with risk-taking behavior. Indeed, rats with increased levels of oxytocin presented an increased risk-taking behavior, reinforcing the neuroprotective effects of oxytocin.

Impressively, these alterations were associated with large reductions in biochemical markers of the glutamatergic synapse in the hippocampus (ventral and dorsal) and in prefrontal cortex, which were exclusively seen in males. PRS decreased protein levels of mGlu5 receptors, and the GluN1, subunit of NMDA receptors in male rats. A similar scenario has been reported in adult rats, in which PRS reduced hippocampal mGlu5 receptors only in males (Zuena et al., 2008). mGlu5 and NMDA receptors are physically linked by a chain of scaffolding proteins, and they functionally interact in the induction of activity-dependent synaptic plasticity (Alagarsamy et al., 1999; Rosenbrock et al., 2010). This suggests that PRS impairs the receptor substrates of synaptic plasticity in aged male rats. Interestingly, the effect of PRS on mGlu2/3 protein levels in aged rats was not gender-dependent, as shown by a reduction in the

ventral hippocampus and prefrontal cortex of both sexes. Again, this finding was similar to what reported in the past in whole hippocampus of adult rats, where PRS reduced mGlu2/3 receptor protein levels in both sexes (Zuena et al., 2008). Activation of the mGlu2/3 receptors with the agonist, LY354740, induces an increased risk-taking behavior in male rats (Schoepp DD et al., 2003; Linden AM et al., 2005), and mGlu2/3 receptors are targets for anxiety and stress disorders in humans (Swanson et al., Nat. Rev. Drug Disc., 2005). Here, the reduced mGlu2/3 receptor expression was associated with a decreased exploratory behavior in PRS rats of both sexes. mGlu2/3 receptors are preferentially localized in presynaptic terminals and are known to negatively regulate glutamate release (Nicoletti et al., 2011). Using adult rats, we have found that PRS leads to a reduction in glutamate release in the ventral hippocampus (Marrocco et al., 2012). Hence, the reduction of mGlu2/3 receptor expression seen during early life (Laloux et al., 2012), in the adult life (Zuena et al., 2008), and during ageing (present data) could represent an allostatic compensatory mechanism, which operates across the entire lifespan. One the most interesting findings related to a possible hypofunction of the glutamatergic synapse in PRS males was the reduction of NMDA and AMPA receptor subunits. NMDA and AMPA receptors are ligand-gated ion channels that are involved in the induction and expression of long-term potentiation (LTP) and long-term depression (LTD) of excitatory synaptic transmission, respectively (Paoletti et al., 2013; Diering and Huganir, 2018). Consistently with our results, Derks and collaborators reported that early life stress impairs the development of synaptic plasticity in the CA1 hippocampus in a sex-dependent manner, with males being more vulnerable (Derks et al., 2016). Furthermore, a decrease in GluN2B following early life stress has been reported in the hippocampus (Lesuis et al., 2019). We also found an increase in the expression of the cystine-glutamate antiporter (xCT) in the ventral hippocampus and prefrontal cortex of female rats. Interestingly, xCT protein levels in PRS female rats differed between the dorsal and ventral hippocampus. While xCT protein levels were increased in the ventral hippocampus, they were decreased in the dorsal hippocampus of female with respect to male PRS rats. xCT in the hippocampal dentate gyrus has been implicated in mechanisms of resilience to stress (Nasca et al., 2017). Glutamate homeostasis differs between the ventral and dorsal hippocampus (Marrocco et al., 2012; Nasca et al., 2017), and, therefore, the differential expression of xCT we have found in PRS rats could help explain the greater involvement of the ventral hippocampus in the "pathological" phenotype triggered by PRS. Glutamatergic neurotransmission depends also of synaptic vesicle-related proteins that are involved in glutamate release (Augustin et al., 1999; Südhof, 2012). Synaptic vesicle-associated proteins were decreased by PRS in the ventral

hippocampus and striatum of male rats, whereas no changes were found in female PRS rats. The hypofunction of glutamate transmission observed in the ventral hippocampus of aged male PRS rats is in agreement with previous results obtained in adult male PRS rats (Marrocco et al., 2012; Marrocco et al., 2014, Mairesse et al., 2015; Morley-Fletcher et al., 2018) supporting the hypothesis that alterations in the glutamate synapse in the ventral hippocampus lies at the core of the programming triggered by PRS in adult male rats.

Glucocorticoids regulate glutamate transmission via mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (Popoli et al., 2011; Karst et al, 2005; Di et al., 2009). Furthermore, it has been described an underlying mechanism by which glucocorticoids, acting via MRs, decrease resilience to stress via downregulation of mGlu2 receptors (Nasca et al., 2014). In this study we showed that PRS decreased GR and MR protein levels in the ventral hippocampus of male rats. Activation of GRs by glucocorticoids mediates the negative feedback of the HPA axis. Hence a decreased GRs and MRs expression could impair the functioning and regulation of the HPA axis. Accordingly, prolonged corticosterone secretion following acute stress associated with reduced GR/MR protein levels in the hippocampus has been reported in male adult PRS rats (Maccari et al., 1995; Maccari et al., 2017). Furthermore, Vallee and collaborators (1999) demonstrated that circulating glucocorticoids levels of PRS middle-aged rats were similar to the levels of old control rats, suggesting that PRS accelerated the age-related dysfunction of the HPA axis. These data raise the attractive possibility that the impairment of the HPA axis in PRS rats may be involved in programming a hypofunction of the glutamatergic synapse until aging. Oxytocin, which is involved in maternal care (Batz et al., 2010; Champagne et al., 2001; Gatta et al., 2018) acts as an anti-stress hormone (Windle et al., 1997, 2004; Bartz et al., 2011; Mairesse et al., 2015). Although plasma oxytocin levels were unchanged by PRS and sex, we observed an increase of oxytocin receptors in the prefrontal cortex in female PRS rats, which is indicative of an increased oxytocinergic transmission. Brain derived neurotrophic factor (BDNF) is another key factor in stress response. BDNF-mediated signaling is involved in the structural effects of stress and plays an important role in dendritic remodeling (Magariños et al., 2011; Pawluski et al., 2012). Similarly to oxytocin, BDNF has anti-stress effects since overexpression of BDNF occludes effects of chronic stress (Lakshminarasimhan and Chattarji, 2012) and chronic stress decreases BDNF levels (Shou-Sen Shi et al., 2010). Furthermore, BDNF expression is influenced by maternal separation early in life (Suri et al., 2013). PRS also increased glial fibrillary acidic protein (GFAP) levels in females as compared to PRS males in the dorsal

hippocampus and reduced GFAP levels in PRS males with respect to unstressed males. It has been previously shown that GFAP levels were increased in adult female compared to male rats (Zuena et al., 2008). GFAP is an astrocyte-specific intermediate filament protein (Norton et al., 1992; Middeldorp and Hol, 2011), which increase during reactive astrogliosis associated with neurodegeneration. Changes in BDNF and GFAP observed in male and female PRS rats raise the possibility that early life stress shapes vulnerability to age-related neurodegeneration in a sex-dependent manner.

In order to have a global picture of the overall proteomic profile, we performed multidimensional analyses on collected sets of proteomic data. This revealed a dysmasculinization profile of the glutamatergic synapse and synaptic vesicles related proteins in the ventral hippocampus, dorsal hippocampus and prefrontal cortex, the main brain regions related to stress (McEwen et al., 2016), but not in the striatum. The stress and anti-stress markers showed a dysmasculinization only in the dorsal hippocampus showing a prominent role of glutamatergic synapses than stress system. The dysmasculinization induced by PRS is explained, at least in part, by the decrease of testosterone and increased of estradiol levels in males in the dorsal hippocampus. This is in agreement with earlier works of Ward (1972; 1980) showing decreased testosterone during the fetal period. Interestingly, testosterone reversed the age-related increase in GFAP (Day et al., 1998), which was associated with lower testosterone concentrations in older rats (Nichols et al., 1993). Here, this is particularly true in PRS aged females that have reduced testosterone and an increased GFAP. Furthermore, we showed that aromatase, the enzyme converting testosterone into estradiol, was respectively upregulated in the dorsal hippocampus of PRS males and downregulated in PRS females in agreement with the reduced levels of estradiol in female PRS rats. Although, specific patterns of dysmasculinization have already been reported in fetal and adult life at physiological, and behavioral level in the past decades (Ward, 1972, 1984; Becker and Kowall, 1977; Dahlöf et al., 1977; Dörner et al., 1983; Ward and Weisz, 1984; Morgan and Bale, 2011), our results show that PRS programs a dysmasculinization of the glutamatergic system also during aging. Instead, lower levels of pro-inflammatory cytokine IL-6, higher levels of BDNF and GFAP may protect females against the detrimental consequences of PRS.

Conclusion. Our findings provide the first evidence that long-term programming effects of PRS are strictly sex-dependent and lead to a dysmasculinization of the glutamatergic synapse during aging, reinforcing the idea that early stressful events have long-lasting effects on brain development. The neuroprotective effects against PRS displayed by aged females resonates

with a recent study in human showing that stressors in pregnant women are associated with male vulnerability and poor fetal outcomes (Walsh et al., 2019). Because the aged population is progressively increasing studies aimed at understanding how perturbations of the early life environment, shape the vulnerability to age-related disorders could be very helpful for the quality of life of our modern society. Our data may provide a first step into this direction.

Conflict of interest

The authors declare no conflict of interest

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Supplementary figures

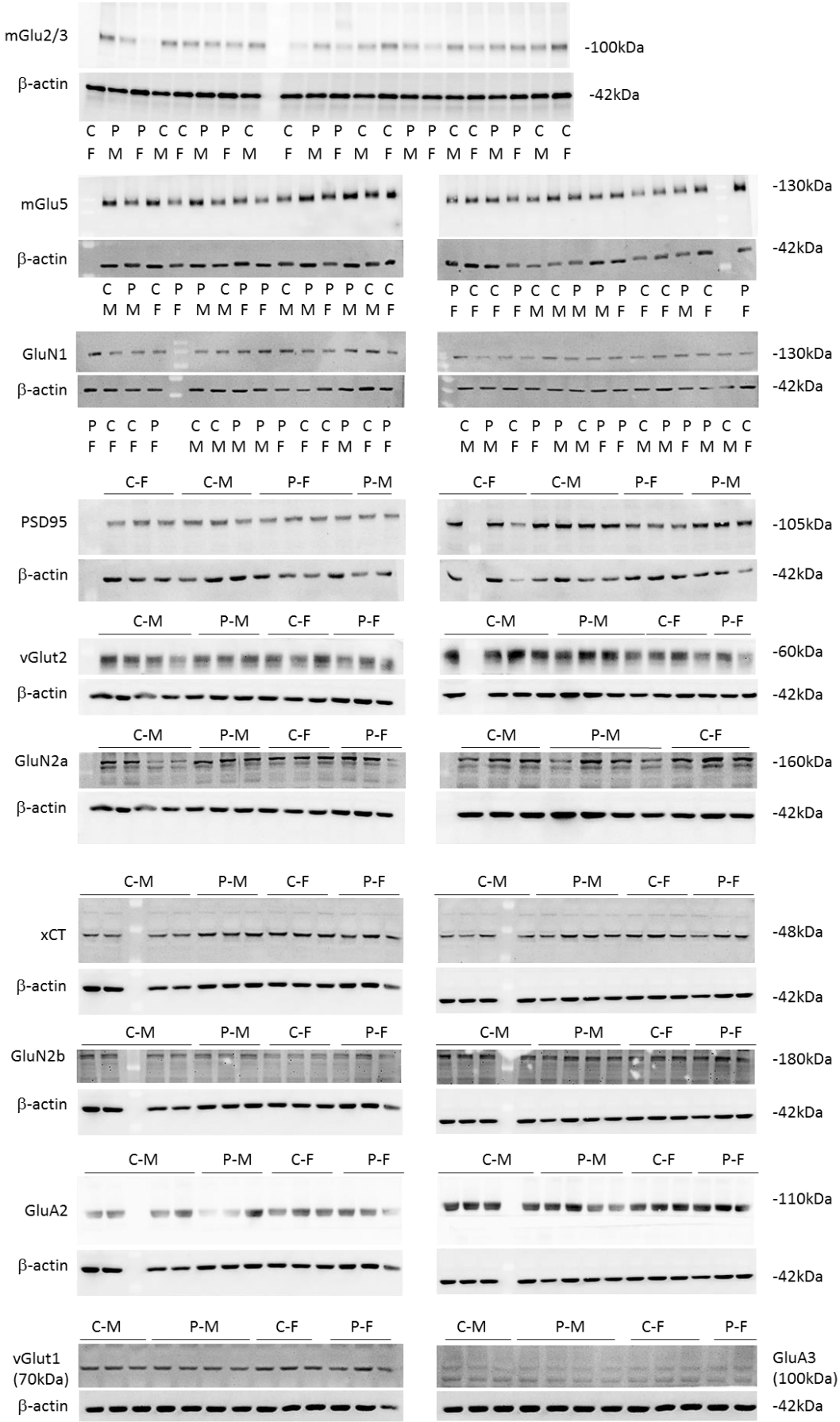

Fig S1a. Immunoblots glutamatergic proteins in the *ventral hippocampus* of aged male female control and PRS rats

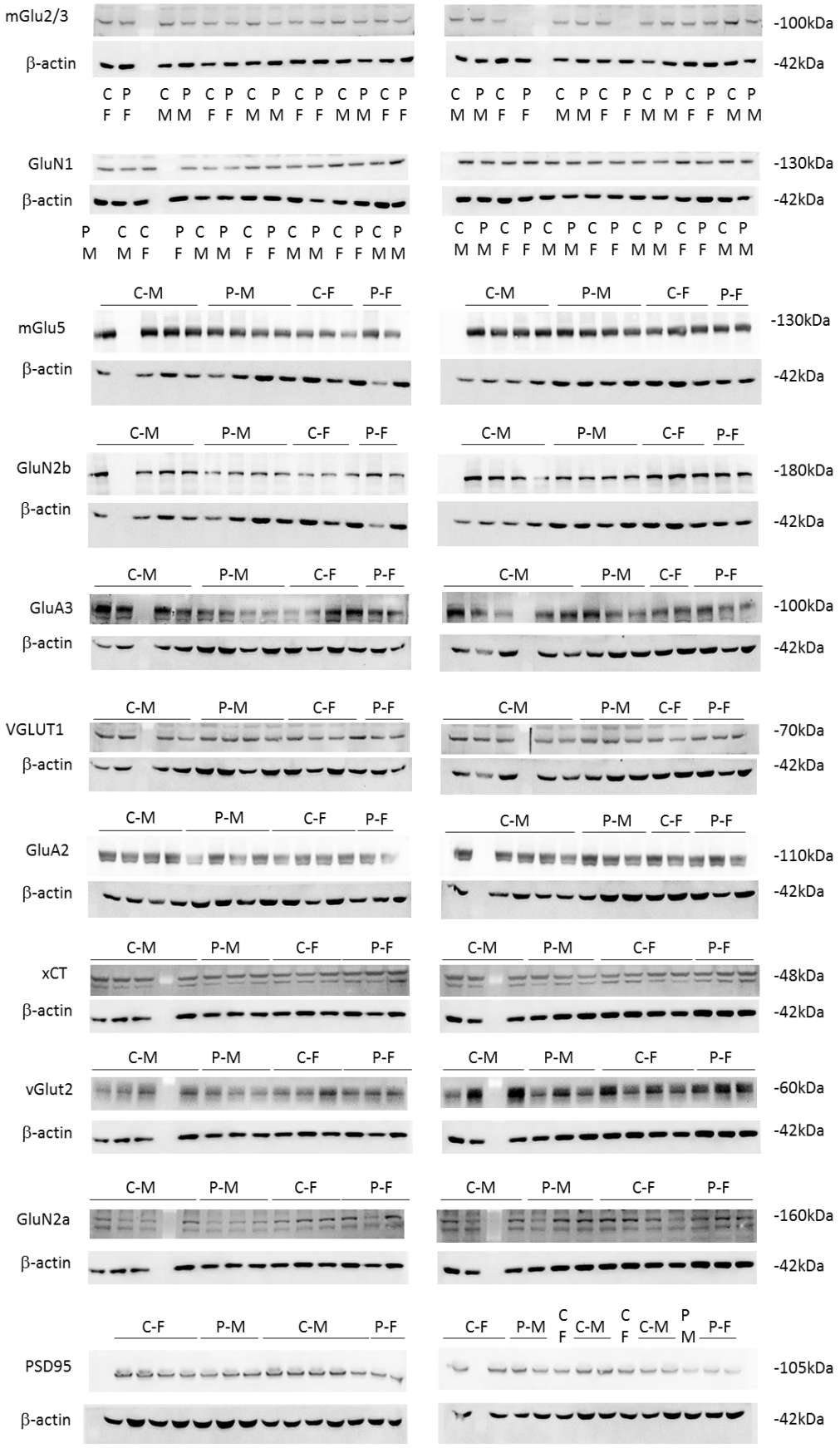

Fig S1b. Immunoblots glutamatergic proteins in the *dorsal hippocampus* of aged male female control and PRS rats

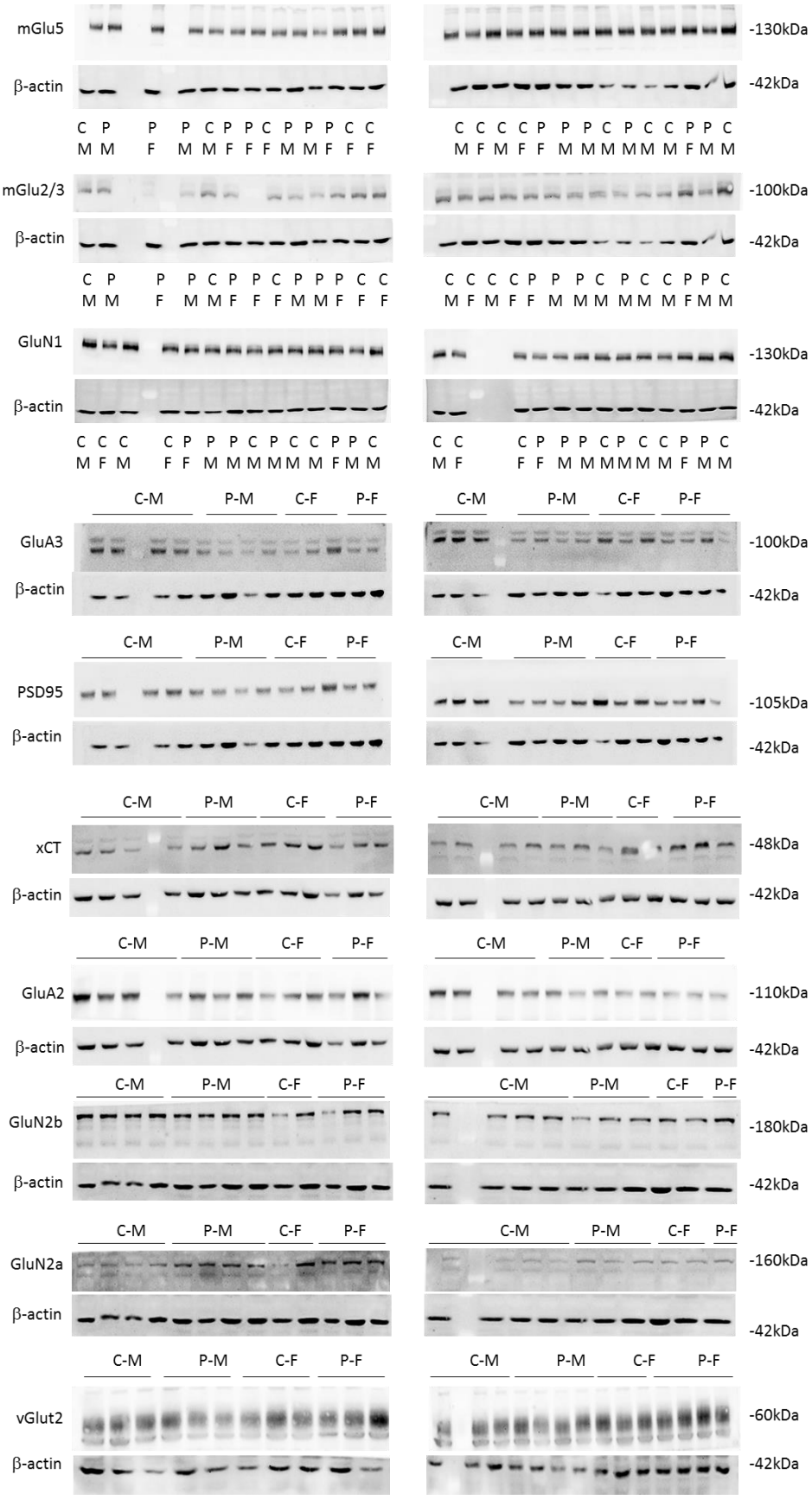

Fig S1c. Immunoblots glutamatergic proteins in the *prefrontal cortex* of aged male female control and PRS rats

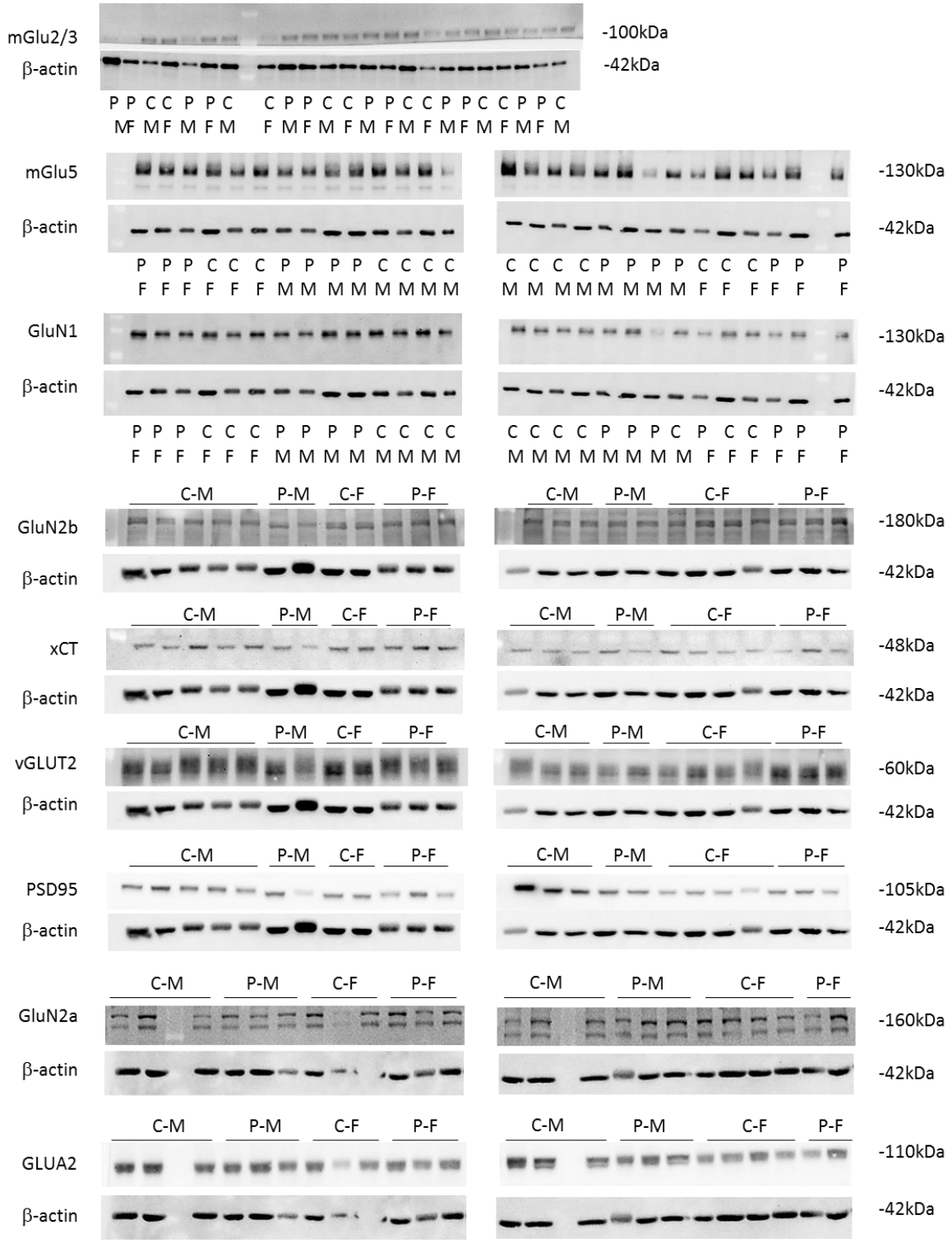

Fig S1d. Immunoblots glutamatergic proteins in the *striatum* of aged male female control and PRS rats

ARTICLE 2

MATERNAL STRESS PROGRAMS AN ACCELERATED AGING OF THE BASAL GANGLIA MOTOR SYSTEM

Running title: ***Early stress induces dysfunctions of aging in rats***

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ABSTRACT

Objective. Early life stress involved in programing stress-related illnesses can have a toxic influence on the functioning of the nigrostriatal motor system during aging.

Methods. We examined the effect of perinatal stress (PRS) on neurochemical, electrophysiological, histological, neuroimaging and behavioral correlates of striatal motor function in adult and old male rats.

Results. Adult PRS offspring rats showed reduced dopamine (DA) release in the striatum associated with a reduction of TH+ cells and DA transporter (DAT), with no loss of striatal dopaminergic terminals as assessed by PET analysis with ^{18}F-DOPA. Striatal levels of DA and its metabolites were increased in PRS rats. In contrast, D_2 DA receptor signaling was reduced and A_{2A} adenosine receptor signaling was increased in the striatum of adult PRS rats. This indicates an enhanced activity of the indirect pathway of the basal ganglia motor circuit. Adult PRS rats also showed reduced performance in the grip strength test and in motor learning tasks. In the aged PRS rats there is also a persistent reduction in striatal DA release and defective motor skills in the pasta matrix, and ladder rung walking tests. In addition, in the striatum, old rats showed large increase of SNAP-25 and SYP synaptic vesicle-related proteins and, PRS group only, a reduction of Syntaxin-1 and Rab3a proteins.

Interpretation. The age-dependent threshold for motor dysfunction is lowered in PRS rats. This area of research is underdeveloped and raises the attractive possibility that PRS contributes to the understanding of the way aging diseases are programmed in early life.

Keywords: Perinatal Stress, nigrostriatal development, motor behavior, dopamine, adenosine receptors, synaptic proteins, integrated longitudinal study, aging

INTRODUCTION

The comorbidity between motor and stress-related disorders is well established (Laureti et al., 2016; Suguma et al., 2016), but the nature of this association remains largely unexplored. In particular, little is known on how early life stress, which predisposes to stress-related disorders, affects the development of the nigrostriatal system and represents a risk factor for age-related disorders. The neostriatum, the major input station of the basal ganglia motor circuit, is connected to output stations (the internal globus pallidus and the *substantia nigra* pars reticulata) through the direct and indirect pathways. An increased activity of the indirect pathway involving D_2 dopamine (DA) and A_{2A} adenosine receptors, as occurs in Parkinson's disease (PD), enhances the inhibitory control of the output stations on thalamocortical neurons. In contrast, an increased activity of the direct pathway involving D_1 receptors, leads to an exaggerated motor activity as it occurs in L-DOPA-induced dyskinesias (Conn et al., 2005; Cenci et al., 2009; Calabresi et al., 2014). Not surprisingly, most of the studies on stress and DA have focused on the mesolimbic system. However, changes in the activity of the direct and indirect pathways of the striatal motor circuit have been reported after exposure to uncontrollable stress (Clark et al., 2014), and changes in striatal DA levels are observed in response to chronic stress in adult rats (Ida et al., 1982). Interestingly, early life stress causes changes in DA turnover in the striatum, nucleus accumbens, and prefrontal cortex (Fride and Weinstock, 1988; Alonso et al., 1997), leading to a delay in motor development (Barlow et al., 1978).

The rat model of perinatal stress (PRS), in which exposure of pregnant dams to restraint stress reduces maternal behavior (Gatta et al., 2018), has face, construct, and pharmacological validity as an epigenetic model of stress-related disorders (Maccari et al., 2014). PRS alters the developmental trajectory of the offspring, causing long-lasting neurochemical, endocrine, and behavioral alterations (Maccari et al., 2017). These alterations are associated with a large reduction in glutamate release and in the expression of synaptic vesicle-associated proteins in the ventral hippocampus (Marrocco et al., 2012; Morley-Fletcher et al. 2018). PRS also affects the mesolimbic dopaminergic system, increasing amphetamine self-administration and hedonic sensitivity to natural reward (Deminiere et al., 1992; Reynaert et al., 2016). There are only a few studies on how early life stress programs the activity of the basal ganglia motor circuit in the adult life. For example, PRS was shown to affect the symmetry of D_2 receptor expression in the medial caudate/putamen (Adrover et al., 2007), and to restrain haloperidol-induced catalepsy and enhance apomorphine-induced stereotypies (Marrocco et al., 2013).

However, no studies have ever been performed in old rats even if it is well known that there exists a decline of mobility with age and, age is an established risk factor for motor disorders as observed in the PD. These observations gave us the impetus to examine whether PRS causes long-lasting changes in the striatal motor programming, and whether some of these changes persist during aging.

MATERIALS AND METHODS

Experimental design

The experimental timelines are shown in **Figure 1**. After PRS procedure consisting in the restraint stress of the pregnant dams and reduced maternal behavior in the first *postpartum* week, behavioral, and biochemical measurements in the striatum of adult (3-5 months old) and aged (21 months old) male progeny were studied. All experiments followed the rules of European Communities Council Directive 86/609/EEC. The Local Committee CEEA-75 (Comité d'Ethique en Experimentation Animale Nord-Pas de Calais, 75) approved the experimental protocol.

Animals

Ninety nulliparous female Sprague–Dawley rats, weighing approximately 250 g, were purchased from Charles River (France) and housed under standard conditions with a 12 h light/dark cycle. After a group housing (5 females/cage) for two weeks, each female was individually housed for one week with a sexually experienced male rat. Following that, a gain of at least 10 grams was considered as index of pregnant status.

Stress procedure

The stress procedure was performed on 3 separate sets of breeding, each set using 30 females (15 control and 15 stressed dams). PRS, i.e., the adult offspring of dams exposed to multiple episodes of restraint stress during pregnancy causing reduced maternal care, was obtained according to our standard protocol (Maccari et al., 1995) and showed in the **Fig. 1**. Briefly, from day 11 of pregnancy until delivery, pregnant females were subjected to restraint in a transparent plastic cylinder and exposed to bright light during three daily sessions of 45 min. Control pregnant females were left undisturbed in their home cages and weekly handled. After weaning, only male offspring from litter with a balanced sex ratio were used for the experiments. Animals were housed in-group of two or three and maintained under similar environmental condition all life span.

Behavioral studies

Two-way active avoidance test

Motor learning and locomotion rats were measured through a two-way active avoidance (AA) test by means of a shuttle-box. AA task requires learning that an explicit conditioned stimulus (CS, light) precedes the delivery of a negative unconditioned stimulus (US, foot-shock).

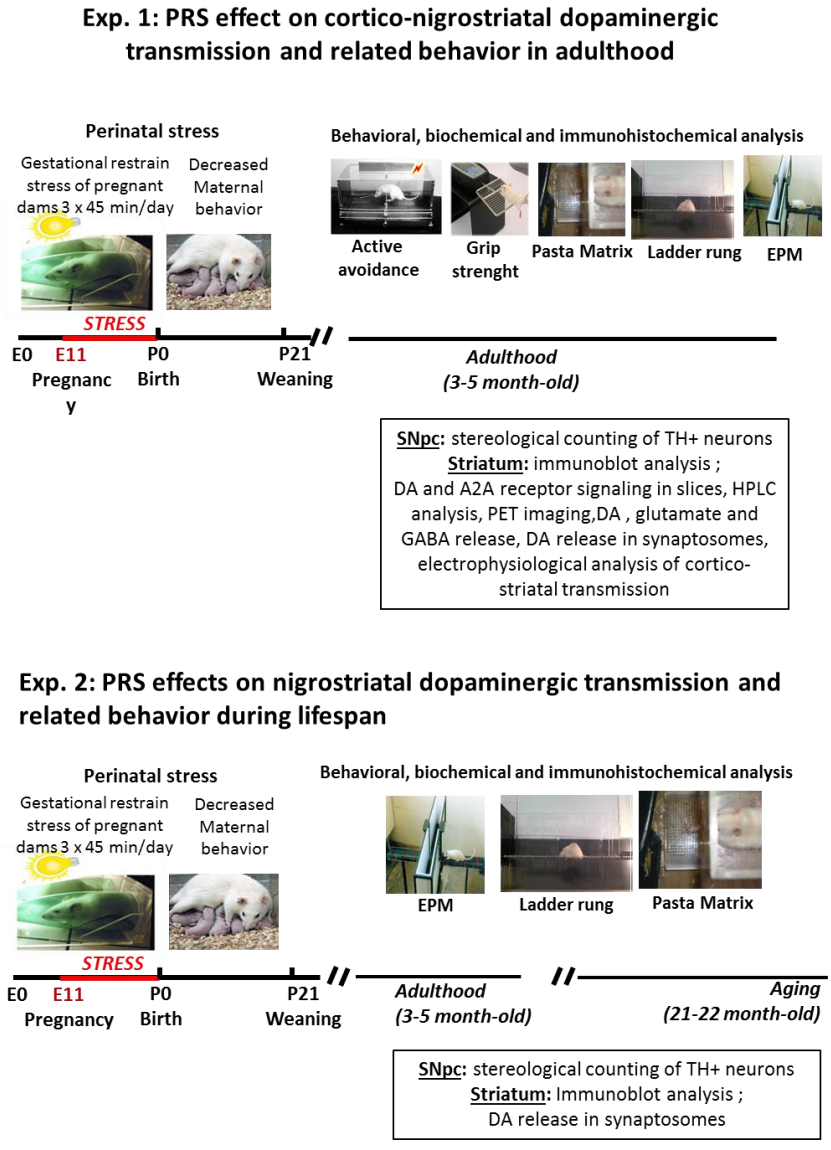

Figure 1. Experimental design and timeline of experiment 1 and 2
Induction of PRS and analysis of maternal behavior in the first *postpartum* week were followed by behavioral, and biochemical measurements in the adult (3-5 months old) and old (21-22 month-old) male progeny as indicated. D_1R, D_2R, $A_{2A}R$ = D_1, D_2, and A_{2A} receptors; SNpc = *substantia nigra* pars compacta.

A shuttle box (40x10x15 cm) equipped with transparent cover, light bulb, stainless-steel grid at the floor and two communicating compartments was used. Rats were subjected to one AA session (duration, 30 min; 60 avoidance trials) for 5 consecutive days. Each trial consisted of 22 sec of darkness followed by a 4 sec light signal (CS) presented in one compartment and subsequently 4 sec of light associated to an electric foot-shock (0.2 mA, 25 sec) in the same compartment (US). The number of conditioned responses in the AA (crossings occurring within 8 sec of CS), the average escape latency to cross to the other compartment after the beginning of each CS, and the number of inter-trial crossings between the two compartments were automatically recorded (Ugo Basile). Foot-shock sensitivity was evaluated by measuring pain thresholds with increasing current intensities (0-0.6 mA) and the minimal intensity eliciting vocalization and jumping was retained as the score. Rats failing to squeak were given the maximum score of 0.6 mA.

Grip strength test

Grip strength of adult and PRS rats was measured using an automated grip strength meter (BiosebLab, Vitrolles, France). The apparatus consisted of a single grip crossbar linked to a single strain gauge connected to a printed circuit chip for a direct digital readout of measurements. Rats per group were trained to grasp the grid and subsequently tested for three consecutive days. Rats were held by the tail and their hind-limbs over the mesh grid of the meter, and once the forepaws, but neither hind-limbs, were both firmly grasping the grid, rats were pulled by the tail along the axis of the force sensor until they were unable to retain their grip. Three measurements per trial were then averaged for each rat on each day for analysis.

Pasta matrix reaching test

Fine motor skills of adult and aged control and PRS rats were assessed in the pasta matrix-reaching task (adapted from Ballermann et al., 2001). The experimental apparatus consisted of a transparent box in plexiglass (14x35x35 cm), which housed the animal that included a 4 mm wide vertical slit on one side. A shelf made of 230 holes, inside which pieces of raw spaghetti (Spaghettini Barilla # 3) were vertically inserted, was placed next to the vertical slit. One week before the test, rats were individually housed into single cages and given the pasta pieces in their home cage *ad libitum* for 1 week in addition to their food pellets. On day 1, rats were food-deprived at 7:00 pm for 24 hours and then received the pasta pieces in their home cage from 7:00 pm to 9:00 pm the day after (day 2). On day 3, rats were habituated to the experimental apparatus without spaghetti and left undisturbed from 7:00 pm to 9:00 pm. On day 4, rats were placed into the experimental apparatus containing spaghettis and left undisturbed from 7:00 to 9:00 pm. The coordinates and total number of pasta pieces retrieved on day 4 were recorded by observing the pasta shelf at the end of the session. An Excel matrix obtained from the number of retrieved spaghettis per box was created to visualize the results that were plotted using a heat-map.

Elevated Plus Maze

Risk-taking behavior of PRS or control adult and aged progeny was assessed in the elevated-plus maze (EPM) (Pellow et al., 1985; Morley-Fletcher et al., 2018). Briefly, the test was performed between 1.00 pm and 4.00 pm, lasted for 5 min and began with the placement of the rat in the center of the maze with the head facing a closed arm. For adult animals we used a standard EPM apparatus with closed and open arms 10 x 10 cm, while for aged animals we used a custom-made EPM apparatus as described by Vallée et al. (1999), with closed and open arms 20 x 20 cm. The luminosity of closed arms was about 25 lux and the luminosity of open arms was about 50 lux. Behavior was recorded on-line by a video camera and manually scored by a trained observer blind to animals' conditions (PRS and control) by using a specific software (The Observer®, Noldus, The Netherlands). The time spent in open and closed arms recorded by a video camera and the percentage of time spent in open arms was calculated.

Ladder rung walking test

Fine motor skills of aged control and PRS rats were assessed in the horizontal ladder rung walking test (Metz and Whishaw, 2002). The apparatus consisted of side walls made of clear Plexiglas and metal rungs (3 mm diameter), which could be inserted to create a floor with a minimum distance of 1 cm between rungs. The sidewalls were 1 m long and 19 cm high measured from the height of the rungs. The ladder was elevated 30 cm above the ground with a refuge (home cage) at the end. Varying the position of the metal rungs modified the difficulty of the task. A regular pattern of the rungs allowed the animals to learn the pattern over several training sessions and to anticipate the position of the rungs. After the training sessions an irregular pattern has been made in order to analyze how rats managed to cross the ladder with an increase of the difficulty. The behavior of the rat on the ladder was recorded using two video cameras. One video camera was placed in front of the first half of the ladder in a slight ventral angle and the other was placed in front of the second half of the ladder in a slight ventral angle in order to analyze precisely the misplacement of the rat's paw. Following the video recording, the foot fault scoring was analyzed. The score of one was attributed when one rat's paw was misplaced and the score of two was attributed when one rat's paw deeply slipped or missed. Then, the percentage of error increased between the last training and the test with the irregular pattern has been calculated as follow: (error score irregular pattern-error score last training)/ error score last training *100.

Electrophysiology

The preparation and maintenance of coronal corticostriatal slices have been described previously (Cacace et al., 2017). Whole-cell patch-clamp recordings were performed as previously described from medium spiny neurons (MSNs) (Bagetta et al., 2012) visualized using infrared differential interference contrast microscopy in the dorsal striatum (Eclipse FN1, Nikon) (Bagetta et al., 2011).

Induction of long-term depression (LTD) of excitatory transmission in the striatum

After recording evoked EPSPs of stable amplitudes for at least 10 min, to induce LTD, a high-frequency stimulation (HFS) protocol consisting of three trains of 3 seconds (20 seconds interval) was delivered at 100 Hz. LTD plots were obtained by averaging the peak amplitudes of EPSPs every minute. Changes of EPSP amplitude induced by stimulation protocols were expressed as a percentage of the baseline, the latter representing the normalized EPSP mean amplitude acquired during a stable period (10–15 min.) before delivering stimulation.

[^{18}F]-DOPA PET imaging procedure

Rats were taken to imaging facility (Univ Lille, Medical Campus) the night before imaging and kept at room temperature with free access to food and water. An i.p. injection of carbidopa (10 mg/kg, Sigma-Aldrich, Saint-Quentin Fallavier, France) was administered 30-60 min prior to radiotracer administration.

Each rat was anesthetized with isoflurane (5% for induction and 1.5-2.5% for maintenance in 100% O_2 at a flow rate of 1 L/min) using a nose cone and positioned so that the center of the field of view was 2 mm caudal to the line between the lateral edges of the eyes, with the help of a laser alignment device within to the scanner. A CT scan (80KV and 500 mA) was run right before the rat moved into the PET field of view. A i.v. bolus injection of [^{18}F]-DOPA (30±5 MBq; 400-900 µl in volume, IBA-CisBio, Saclay, France) was administered via the caudal vein. PET scanning was initiated at the onset of [^{18}F]-DOPA administration. Total scanning time was 55 min. Data from the scanner were formatted into 30 frames, OSEM2D reconstructed and corrected for scatter and attenuation. Counts detected by the scanner were converted into percentage of injected dose/g by use of IRW software (Inveon Research Workflow, version 3.0, Siemens). This software enables PET-CT co-registration and allows imaging realignment with brain rat T2-weighted MRI. ROIs were manually drawn on axial and coronal views of the MRI scan, then transferred to the PET images. A background ROI was also drawn outside the brain region. In all cases, the apparent activity in this region reflected the contributions from random coincidence events and from scatter and was found to be very small throughout the course of the studies.

Measurements of catecholamines in the striatum

Analysis of DA, 3,4-dihydroxyphenylacetic (DOPAC) and homovanillic acid (HVA) was performed by HPLC equipped with an autosampler 507 (Beckman Instruments, Fullerton, CA), a programmable solvent module 126 (Beckman), an analytical C18 reverse-phase column kept at 30°C (Ultrasphere ODS 5 mm, 80 Å pore, 250 x 4.6 mm (Beckman), and a Coulochem II electrochemical detector (ESA, Inc., Chelmsford, MA). The holding potentials were set at +350 and –350 mV for the detection of DA, DOPAC and HVA. The mobile phase consisted of 80 mM sodium phosphate, 40 mM citric acid, 0.4

mM EDTA, 3 mM 1-heptansulphonic acid and 12.5% methanol, brought to pH 2.75 with phosphoric acid (run under isocratic conditions, at 1 ml/min).

Microdialysis in freely moving rats

Control male rats and PRS male rats, 5-months old (350-400 g), were used to assess the release of DA in the striatum of freely moving rats by microdialysis. Rats were implanted with microdialysis intracerebral guides (CMA/12 Guide Cannula, CMA/Microdialysis, Stockholm, Sweden), under isoflurane (5% for induction and 2% for maintenance) anesthesia, in a Kopf stereotaxic frame. The site of implantation was the left striatum at the following coordinates: 0.7 mm anterior to the bregma, 2.5 mm lateral to the midline, 3.5-5.5 mm ventral from the surface of skull, according to the atlas of Paxinos and Watson (1998). After surgery, each rat was housed in a cage in a temperature-controlled environment on a 12-hour light/dark cycle, with water and food *ad libitum*, and allowed to recover for four days before the experiment. On the evening before the experiment, a concentric vertical microdialysis probe (2 mm long and 0.24 mm in outer diameter having a cuprophane membrane with a molecular cut-off of 6,000 Daltons, CMA/12 Microdialysis Probe, CMA/Microdialysis) was inserted into the intracerebral guide, after removing a dummy, and the rat was transferred to a plastic bowl cage with a moving arm (CMA/120 System for Freely-Moving Animals, CMA/Microdialysis) with free access to water and food. The probe was perfused continuously with artificial cerebro-spinal fluid containing 150 mM NaCl, 3 mM KCl, 1.7 mM $CaCl_2$, 0.9 mM $MgCl_2$, at a flow rate of 1.5 ml/min, using a microinjection pump (Bioanalytical System Inc., West Lafayette, Indiana, USA). On the following morning, 45 ml (30 min) of consecutive perfusate sample fractions were continuously collected by a fraction collector (CMA/142 Microfraction Collector, CMA/Microdialysis). After 4 sample fractions, used to determine the basal levels of DA, veratridine (100 mM) was perfused for 20 min and sample fractions were collected for the next 2 hours. Changes in the perfusion medium were performed by a liquid switch (CMA/111 syringe selector, CMA/Microdialysis) to avoid any interruption of flow. The analysis of DA was performed as above.

Measurements of neurotransmitter release from superfused striatal synaptosomes

Striatal samples were homogenized in 10 volumes of 0.32 M sucrose containing buffered to pH 7.4 with Tris (0.01 M) and synaptosomes isolated by centrifugation as previously described by Marrocco et al., (2012). The synaptosomal pellet was then resuspended in medium containing (mM): NaCl, 140 KCl, 3 MgSO4, 1.2 CaCl2, 1.2 NaH2PO4, 1.2 NaHCO3, 5 HEPES, 10 mM, glucose, 10 pH 7.2-7.4. Striatal synaptosomes were labeled with [^{3}H]-DA, D-[^{3}H] aspartate or [^{3}H]-GABA. Labeling of dopaminergic terminals with [^{3}H]-DA was carried out in the presence of ascorbic acid, pargyline, and 6-nitroquipazine and desipramine to avoid labeling of serotonergic or noradrenergic terminals, respectively. Release experiment was performed as described previously on Marrocco et al., (2012).

Western blot analysis in the striatum

Immunoblot analysis was carried out under standard conditions (Marrocco et al., 2012) using the following primary antibodies: anti-tyrosine hydroxylase (TH H126, Santa Cruz, 1:1000); anti-D1 receptors (Ab20066, Abcam 1:500), anti-D2 receptors (Ab85367, Abcam, 1:500); anti-DAT (high affinity DA transporter) (ab111468, Abcam, 1:1000); anti-adenosine receptors (sc-13937, Santa Cruz, 1:500), synaptic vesicle-associated proteins: anti-Rab3a (#107111, Synaptic System, 1:2000); anti-Munc18 (#116011, Synaptic System 1:2000; anti-SNAP 25 (sc-136267, Santa Cruz 1:5000); anti-SYP (sc-9116 Santa Cruz 1:8000), anti-syntaxin (sc-13994, Santa Cruz 1:4000). Secondary antibodies directed against rabbit or mouse (Amersham) were used at 1:7500 dilution. After immunoblotting, digitized images of bands immunoreactive for target antibodies and actin were acquired (FUSION®) and the area of immunoreactivity corresponding to each band was measured using the ImageJ imaging software. A ratio of target to actin was then determined, and these values were compared for statistical significance.

Measurement of cAMP formation in striatal slices

The striatum from adult control and PRS rats was dissected and sliced with a McIlwain tissue chopper (350x350 mm). Slices were incubated in Krebs-Henseleit buffer (equilibrated with 95% O_2/5% CO_2 to pH 7.4) for 35-45 min at 37°C under constant oxygenation to allow metabolic recovery. Then, an aliquot (40 mL) of gravity-packed slices was transferred to polyethylene tubes and incubated for 45 min prior to the addition of specific drugs. Slices were then incubated in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 15 min. Slices were then challenged with either SKF-38393 (10 mM), 2-chloro-adenosine (100 mM and 500 mM), forskolin (10 mM) or quinpirole (10 mM) + forskolin (quinpirole was added 2 min prior to forskolin). After the addition of drugs, the incubation was continued for 20 min. The reaction was stopped by adding ice-cold 0.4 N $HClO_4$. Samples were rapidly placed on ice and left at -20°C overnight. On the day of the experiment, samples were sonicated for 10-15 s. Samples were added to 2 N K_2CO_3 and then centrifuged at low speed. An aliquot of supernatant was diluted and then used for cyclic AMP determination. Cyclic AMP formation was quantified using a commercially available ELISA kit (Tema Ricerca, Italy). IBMX, forskolin, SKF-38393 and quinpirole were purchased from Sigma-Aldrich (St. Louis, MO).

TH immunostaining in the *substantia nigra*

Brains were dissected out, fixed in Carnoy solution (ethanol, acetic acid, and chloroform, 6:1:3), and included in paraffin. Tissue sections (15 mm) were incubated overnight with mouse monoclonal anti-TH antibodies (1:200, Sigma, Italy, Milano, cat. T1299) and then for 1 h with secondary biotin-coupled anti-mouse antibody (1:200; Vector Laboratories, Burlingame, CA, BA2000). 3,3-Diaminobenzidine tetrachloride (Sigma) was used for detection.

Stereological cell counting

The number of TH^+ cells in the *substantia nigra* was assessed by stereological technique and optical fractionator using a Zeiss Axio Imager M1 microscope equipped with a motorized stage and focus control system (Zeta axis), and with a digital video camera. The software Image-Pro Plus 6.2 for Windows (Media Cybernetics, Inc., Bethesda, MD) equipped with a Macro was used for the analysis of digital images. The Macro was obtained by Immagine and Computer, Bareggio, Italy. The analysis was performed on 7 sections of 15 mm, sampled every 200 mm in a rostrocaudal extension of the *substantia nigra* from bregma level –4.80 to –6.30. In each stained section the area was identified and outlined at 2.5X magnification. TH+ cells were counted at 100X magnification. For stereological analysis, we used a grid of disectors (counting frame of 50x50 mm; grid size 150x150 mm). The total number of TH-immunoreactive neurons per each rostrocaudal level was computed from the formula $N = \Sigma(n) \times 1/SSF \times 1/ASF \times 1/TSF$, where N is the total number of neurons counted on each disector, SSF (fraction of sections sampled) is the number of regularly spaced sections used for counts divided by the total number of sections through the *substantia nigra* pars compacta (= 1/12); ASF (area sampling frequency) is the disector area divided by the area between disectors [= (2500 μm^2 × dissectors number)/region area], and TSF (thickness sampling frequency) is the disector thickness divided by the section thickness. The total number of TH-immunoreactive neurons in the *substantia nigra* pars compacta is the sum of the total number of TH-immunoreactive neurons per rostrocaudal level.

Statistical analysis

Behavioral, electrophysiological and biochemical data were expressed as the mean ± SEM and analyzed using a parametric analysis of variance (ANOVA) with group as independent variable (CONT *vs*. PRS) and days or treatment as repeated measures (active avoidance and grip strength tests or A_{2A} functionality respectively). When group x day or treatment interaction was present, post-hoc comparisons were performed with Newman-Keul's test. The LTD statistical comparisons between different groups over time were analyzed using two-way ANOVA. The level of significance was set at $p<0.05$.

RESULTS

Experimental design and timeline of experiment 1 and 2 are indicated in **Figure 1.**

Effect of PRS on striatum-related behaviors

In the *active avoidance test* (**Fig. 2A**), PRS reduced the number of conditioned responses as well as the number of box switching (number of conditioned responses, group effect $F_{(1,11)}$= 6.086, p=0.031; number of box switching, group effect $F_{(1,11)}$=17.691, p=0.001; n=6-7 rats/group). Adult PRS male rats also showed impaired striatal motor performance in the *grip strength test* (**Fig. 2B**) i.e. they exhibited reduced strength over three consecutive days of training (group effect $F_{(1,10)}$ = 47.682, p=0.000042; n=6 rats/group). In the *ladder rung walking test* (**Fig. 2C**), we observed an interaction between aging and group ($F_{(1,20)}$=9.956, p=0.0049 n=6 rats/group). By comparing adult and aged rats, we noticed that aging induced defective striatal motor performance in the ladder rung walking test (*Age effect*, $F_{(1,20)}$=28,445, p=0.00004, n= 6 rats/group). Moreover, there was also a group difference (*Group effect* $F_{(1,20)}$=11.004, p=0.0034 n=6 rats/group) as displayed by PRS inducing more errors in both adult and aged rats in the ladder rung walking test when compared to unstressed control rats, although only PRS aged rats made more errors compared to the other groups (Newman-Keuls, p=0.00015). Moreover, PRS affected both age groups in the *pasta matrix reaching test* (**Fig. 2D**), i.e. PRS rats removed a reduced number of pasta pieces associated with decreased extension of the forepaws, as reported in the matrix, with respect to unstressed control rats (group effect, $F_{(1,24)}$=15.184, p=0.001, n=6-7 rats/group). Also, in the elevated-plus maze test (**Fig. 2E**) PRS rats of both ages showed reduced exploratory activity in the open arm indicating reduced risk-taking behavior (group effect, $F_{(1,24)}$=54.838 p=0.001; n=7 rats/group). Thus, PRS induced impairment in striatal behavior both in adult and aged rats.

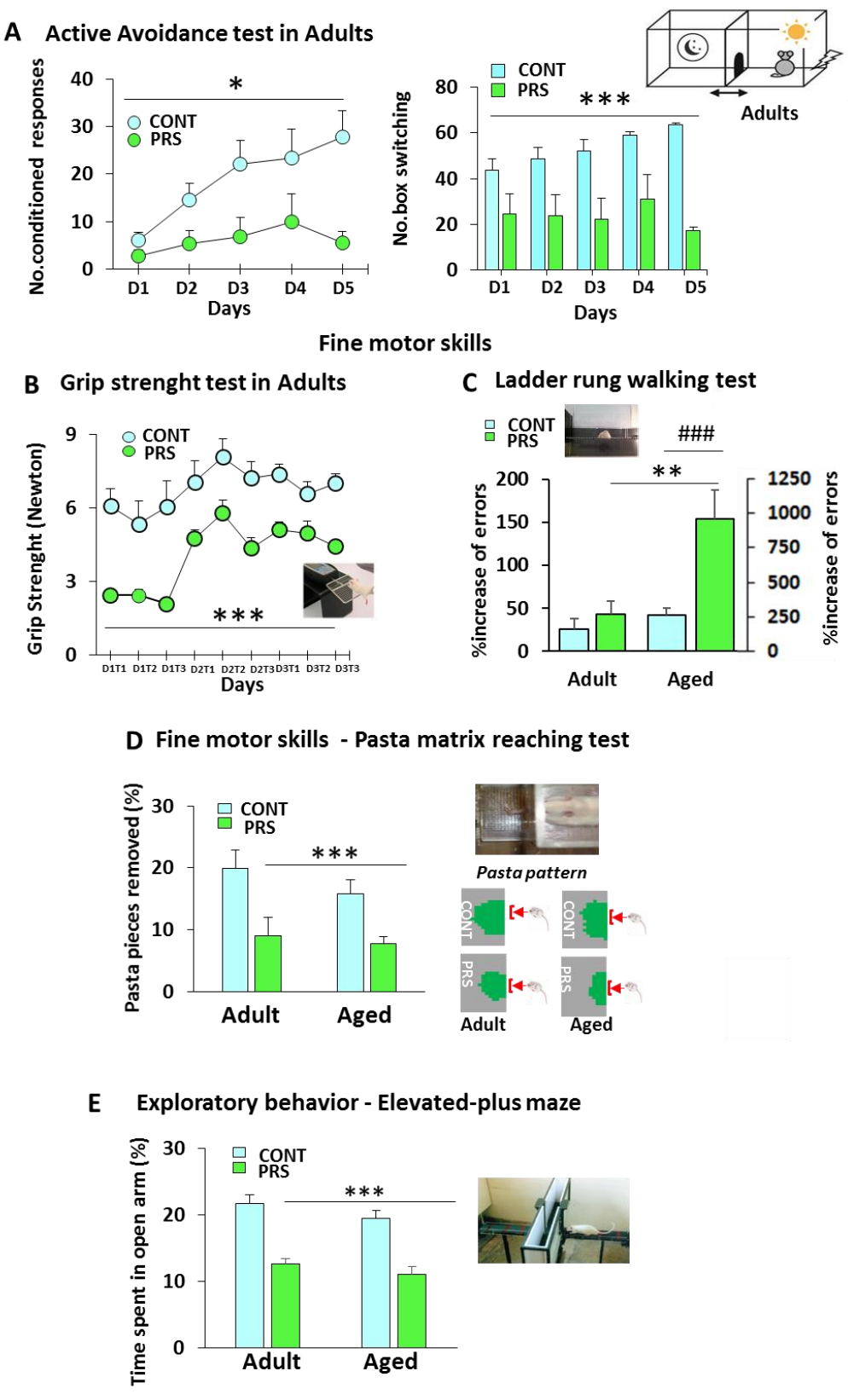

Figure 2. PRS effect on striatum-dependent behaviors in adulthood and during aging
In the *active avoidance test* (Fig. 2A), PRS reduced the number of conditioned responses as well as the number of box switching (n=6-7 adult rats/group). Adult PRS male rats showed a defective striatal motor performance also in the *grip strength test* (Fig. 2B) by displaying reduced strength over three

consecutive days of training (n=6 adult rats/group). Aging induced a defective striatal motor performance only in the *ladder rung-walking test* (Fig. 2C, n= 6 rats/group) where aged rats performed more errors than adults. PRS effect was also reported in both age groups, thus indicating striatal impairment induced by early life stress. In the *pasta matrix-reaching test* (Fig.2D), PRS affected both age groups in the pasta matrix-reaching test, where PRS rats removed a reduced number of pasta pieces with consequent decreased extension of the matrix with respect to unstressed control rats (n=6-7 rats/group). Also, in the *elevated-plus maze test* (Fig. 2E) PRS rats of both ages showed a reduced exploratory of the open arm and consequent risk-taking behavior (n=7 rats/group). Thus, PRS induced a long-lasting impairment in striatal behavior. CONT *vs* PRS * = $p<0.05$; **= $p<0.01$; ***= $p<0.001$; adult *vs* aged ###=$p<0.001$.

Effect of PRS on cortico- nigrostriatal synaptic transmission in adulthood

In the **table 3A**, basal and depolarization-evoked release of glutamate or GABA in superfused striatal synaptosomes did not differ between PRS rats and unstressed controls (group effect: glutamate release, $F_{(1,10)}=0.024$, p=0.87, n=6 rats/group; GABA release $F_{(1,4)}=0.171$, p=0.7, n=3 rats/group). Striatal projection neurons of PRS rats did not show changes in resting membrane potential (group effect, $F_{(1,23)}=0.085$, p=0.772; n=12-13 rats/group) spontaneous excitatory synaptic transmission (measures in Hz: group effect $F_{(1,22)}=0.00571$, p=0.940, n=10-14; measures in pA: group effect $F_{(1,21)}=1.597$, p=0.220, n=10-13 rats/group), NMDA/AMPA ratio-mediated inward currents (group effect $F_{(1,13)}=0.00001$, p= 0.992, n=7-8 rats/group), or LTD (Fig. 3B) of excitatory synaptic transmission in response to high frequency stimulation of the corticostriatal pathway (group x time interaction $F_{(1,18)}=0.729$, p=0.75; n=6 rats/group). Thus, no changes in corticostriatal glutamatergic synaptic transmission and plasticity were found in adult PRS rats. Stereological counting in the *substantia nigra* pars compacta showed a reduction in the number of TH^+ cells of PRS rats (**Fig. 3C)**; (group effect: TH+ cell counting, $F_{(1,13)}= 6.066$, p=0.028, n=7-8 animals/group). In the striatum (**Fig. 3D)**, PRS did not induce changes in TH protein levels. TH immunoblotting, $F_{(1,2)}=0.0147$ p=0.0906, n=6 rats/group), but reduced DAT (*all immunoblots of Fig. 3 are shown in supplementary **Fig. S3***) (group effect: DAT, $F_{(1,12)}=7.542$, p=0.017; n=7 rats/group). *In vivo* micro-PET analysis of [^{18}F]-DOPA uptake in the striatum (**Fig. 3E**) showed no difference between controls and PRS rats (group effect, $F_{(1,6)}=0.455$, p=0.525, n=4 rats/group). In contrast, PRS increased the steady-state levels of DA and its metabolites (DOPAC and HVA) in striatal homogenates compared to controls (**Fig. 3F**, group effect: DA, $F_{(1,20)}=6.403$, p=0.019 HVA, $F_{(1,20)}=6.717$, p=0.017; DOPAC, $F_{(1,20)}=5.216$, p=0.033; n=10-12 rats/group).

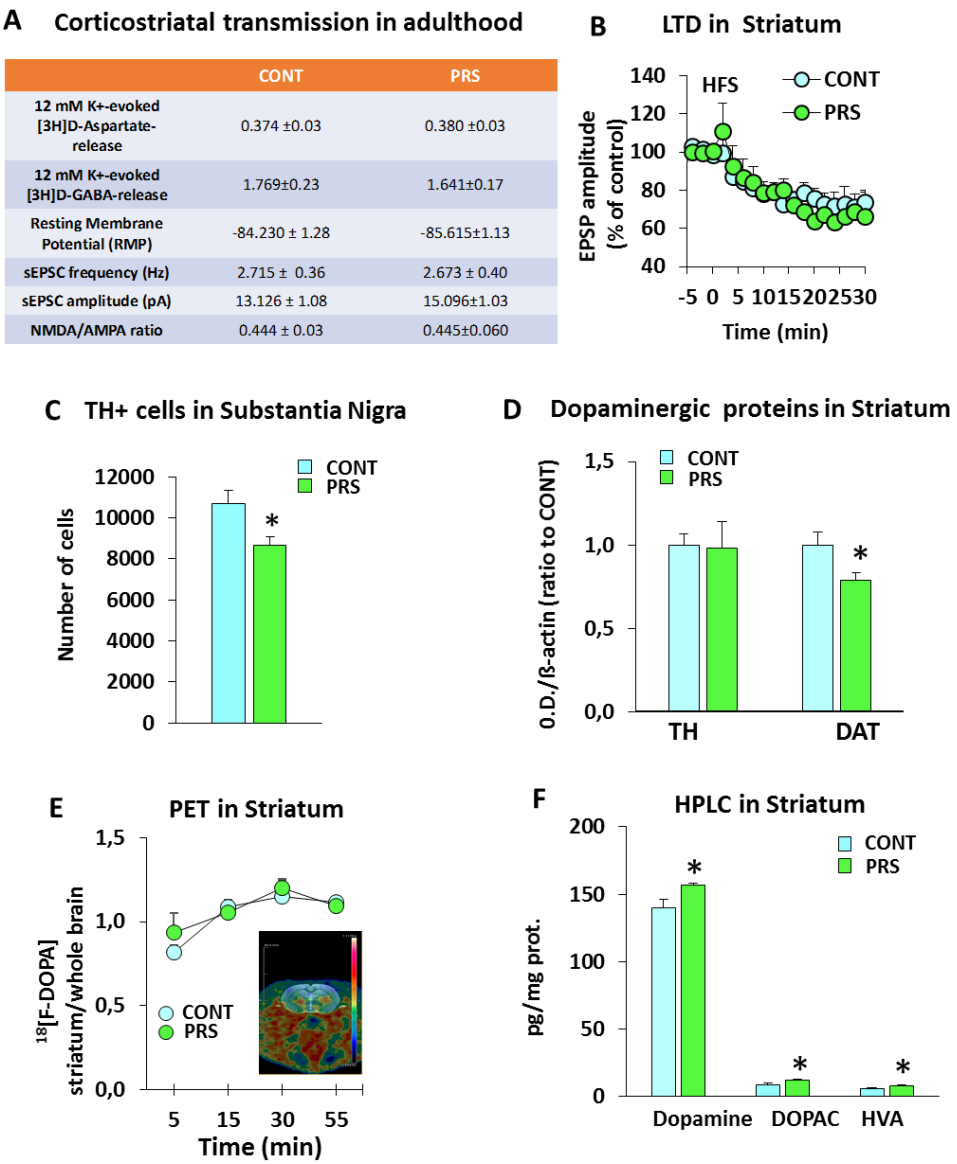

Figure 3. PRS effect on cortico-nigrostriatal synaptic transmission in adulthood

In the table 3A**,** basal and depolarization-evoked release of glutamate or GABA in superfused striatal synaptosomes did not differ between PRS rats and unstressed controls (glutamate n=6 rats/group; GABA release n=3 rats/group). Striatal projection neurons of PRS rats did not show changes in resting membrane potential (n=13 rats/group), spontaneous excitatory synaptic transmission (n=10-15 rats/group), NMDA/AMPA ratio-mediated inward currents (n=7 Ctrl, 9 PRS; Table 3A), and LTD of excitatory synaptic transmission in response to high frequency stimulation of the cortico-striatal pathway (n=6 rats/group) (Fig. 3B). Thus, no changes in cortico-striatal glutamatergic synaptic transmission and plasticity could be detected in adult PRS rats. In the *substantia nigra*, PRS reduced the number of cells expressing the DA synthesizing enzyme tyrosine-hydroxylase (TH), (Fig. 3C; n=7-8 animals/group; but induced no differences in TH protein levels in the striatum (Fig. 3D, n=6 adult rats/group). On the other hand, in the striatum PRS reduced the high affinity DA transporter, DAT (Fig. 3D) (n=7 animals/group). *In vivo* micro-PET analysis of [^{18}F]-DOPA uptake in the striatum (Fig. 3E) showed no difference between controls and PRS rats (n=4 animals/group), suggesting that PRS did not cause detectable changes in the density of dopaminergic nerve terminals in the striatum. In contrast, PRS increased the steady-state levels of DA and its metabolites (DOPAC and HVA) in striatal homogenates (Fig. 3F, n=10-12 rats/group). CONT *vs* PRS * = $p<0.05$

Effect of PRS on nigrostriatal dopaminergic transmission and indirect and direct pathway in adulthood

Microdialysis studies in freely moving rats (**Fig. 4A**), showed a significant decrease in veratridine-stimulated DA release in the striatum of PRS rats compared to controls (group effect, interval 150-240 min post-veratridine, $F_{(1,8)}$=5.651, p=0.0017, n=5 rats/group). A reduction in preloaded [^{3}H]-DA release was also found in purified striatal synaptosomes prepared from PRS rats *vs.* those prepared from control rats, in response to depolarizing concentrations of K+ (**Fig. 4B**) using an up-down superfusion system that prevents indirect effects of endogenous molecules on neurotransmitter release (group effect, $F_{(1,8)}$=36.717, p=0.0003, n=5 rats/group). Together, measurements of DA neurotransmission suggest that PRS reduced DA release in the striatum of adult rats. Immunoblot analysis revealed that PRS downregulated striatal D_1 receptor protein levels (**Fig. 4C**; *all immunoblots of Fig. 4 are shown in supplementary* ***Fig. S4***), with no changes in the levels of D_2 receptors and A_{2A} adenosine receptors, (group effect: D_1R, $F_{(1,10)}$= 9.088, p=0.013; D_2R $F_{(1,10)}$=0.559, p=0.471; A_{2A}R $F_{(1,10)}$=1.312, p=0.278; n=6 rats/group). We extended the analysis to D_1, D_2 and A_{2A} receptor signaling by measuring cAMP levels in striatal slices from PRS and unstressed controls. In spite of the reduction of D_1 receptor protein levels, the ability of the D_1 receptor agonist, SKF-38393 to enhance cAMP formation (treatment effect, $F_{(1,20)}$=30.819, p=0.00002, n=6/rats/group) was unchanged in the striatum of PRS rats (group effect, $F_{(1,20)}$= 0.591, p=0.450, n=6 rats/group) (**Fig. 4D**)**.** In contrast, PRS reduced D_2 receptor signaling (**Fig. 4E**) and amplified A_{2A} receptor signaling (**Fig. 4F**) in the striatum. Accordingly, the extent of inhibition of forskolin-stimulated cAMP formation by the D_2 receptor agonist, quinpirole, was lower in striatal slices prepared from PRS (group effect, $F_{(1,13)}$=15.337, p=0.001, n=7-8 rats/group), whereas stimulation of cAMP formation by the A_{2A} receptor agonist, 2-chloroadenosine, was amplified (Group x Treatment effect, $F_{(2,18)}$=17.877, p=0.001, n=4 rats/group). Thus, the activity of D_2 and A_{2A} was respectively downregulated and upregulated in the striatum of adult PRS rats. Changes in nigrostriatal dopaminergic transmission found in adult PRS rats are summarized in the schematic model in **Fig. 5.**

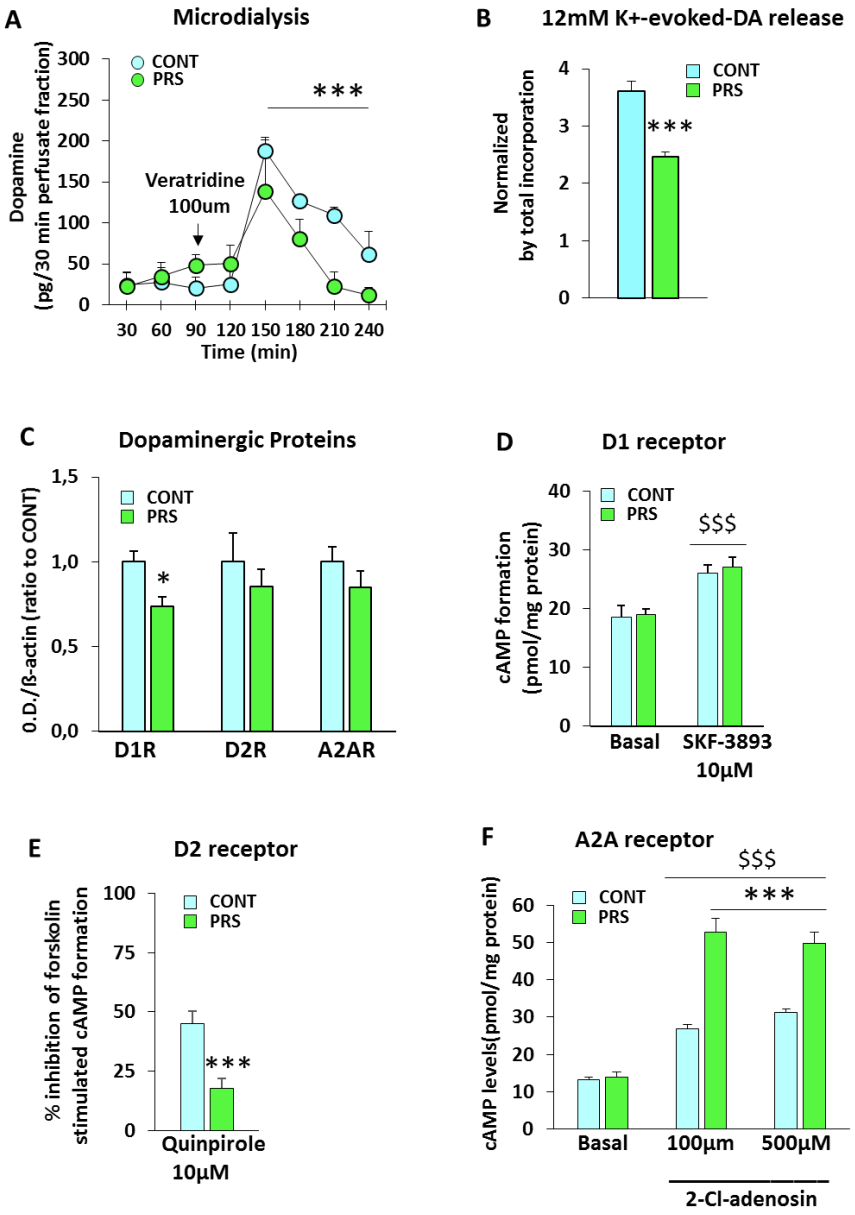

Figure 4. ***Effect of PRS on nigrostriatal dopaminergic transmission and*** **indirect and direct pathway in adulthood**

Microdialysis studies in freely moving rats (Fig. 4A), showed a significant decrease in veratridine-stimulated DA release in the striatum of PRS rats (n=5 rats/group). A reduction in preloaded [^{3}H]-DA release was also found in purified striatal synaptosomes challenged with depolarizing concentrations of K+ (Fig. 4B) using an up-down superfusion system that prevents indirect effects of endogenous molecules on neurotransmitter release (n=5 rats/group). Thus, PRS reduced DA release in the striatum of adult rats. Immunoblot analysis (Fig. 4C) revealed that PRS caused a significant reduction in striatal D_1 receptor protein levels, with no changes in the levels of D_2 receptors and A_{2A} adenosine receptors, (n=6 rats/group). We extended the analysis to D_1, D_2 and A_{2A} receptor signaling by measuring cAMP levels in striatal slices from PRS and unstressed controls. In spite of the reduction of D_1 receptor protein levels, the ability of the D_1 receptor agonist, SKF-38393 to enhance cAMP formation (n=6/rats/group) was unchanged in the striatum of PRS rats (n=6 rats/group) (Fig. 4D)**.** In contrast, PRS reduced D_2 receptor signaling (Fig. 4E) and amplified A_{2A} receptor signaling (Fig. 4F) in the striatum. Accordingly, the extent of inhibition of forskolin-stimulated cAMP formation by the D_2

receptor agonist, quinpirole, was lower in striatal slices prepared from PRS (n=7-8 rats/group), whereas stimulation of cAMP formation by the A_{2A} receptor agonist, 2-chloroadenosine, was amplified (n=4 rats/group). Thus, D_2 and A_{2A} were respectively inhibited and activated in the striatum of adult PRS rats. CONT *vs* PRS * = p<0.05; ***= p<0.001; treatment *vs* basal $$$=p<0.001

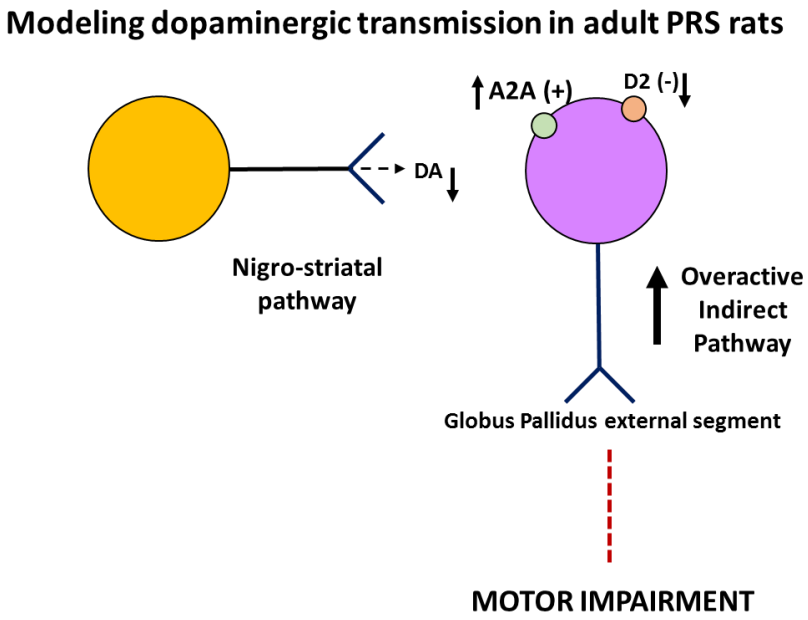

Figure 5. Modeling dopaminergic transmission in adult PRS rats
The net effect of activation of the nigrostriatal pathway is to excite the direct pathway and inhibit the indirect pathway. Adult PRS rats present a reduced striatal dopamine release associated with opposite changes in D_2 and A_{2A} receptor signaling (i.e., a decrease and an increase, respectively) in the striatum. D_2 and A_{2A} receptors form functional antagonistic heteromers that regulate the activity of striato-pallidal neurons of the indirect pathway. The prevalence of A_{2A} over D_2 receptor signaling suggests that the indirect pathway is overactive in the striatum of adult PRS rats. This, combined with the reduced dopamine release, reinforces the inhibitory action of the overactive indirect pathway on the execution of motor program and may account for the lower behavioral performance shown by PRS rats in tests that require a correct functioning of the striatal motor programming.

The comparison adult and aged offspring was studied on the effects of PRS on nigrostriatal dopaminergic transmission.

The absolute number of TH+ cells was reduced by aging in both groups (PRS and unstressed rats) compared to adult rats (**Fig. 6A;** age effect, $F_{(1,26)}$=79.535, p=0.0001; group x age effect $F_{(1,26)}$=3.490, p=0.073; *post- hoc*: CONT *vs.* PRS in adult p= 0.0025, n=7-8 rats/group), *a representative image of TH immunostaining in the substantia nigra of adult and aged rats is shown in the supplementary figure* ***S6a***. ^{3}H-DA release evoked by high concentrations of K+ (12 mM) in striatal synaptosomes was markedly reduced by PRS in both age groups, and, interestingly, DA-evoked release was similar in adult PRS rats and aged unstressed rats. Aging reduced DA release in both unstressed and PRS rats (**Fig. 6B;** group effect, $F_{(1,16)}$=63.324, p=0.00000006; age effect, $F_{(1,16)}$=52.432, p=0.0000002, n=5 rats/group). We then measured dopaminergic proteins in adult and aged rats (**Fig. 6C**). No changes

were reported in striatal levels of TH and $A_{2A}R$ (TH n.s ; $A_{2A}R$ n.s; n=4-8 rats/group), while an age effect was observed for D_1 receptors in both unstressed and PRS groups with reduced levels in old animals in comparison to adult rats (D_1R age effect, $F_{(1,21)}$= 22.796, p=0.0001) and group effect with reduced levels in PRS compared to controls $F_{(1,21)}$=4.785, p=0.04 ; n=4-8 rats/group). Interestingly, in PRS rats, D_1 receptor protein levels were downregulated in both age groups, with a marked effect in adult animals (adult group, CONT *vs.* PRS p=0.037; aged group, CONT *vs.* PRS = 0.315) while DAT was already low in adult PRS rats. Thus, aging reduced DAT protein only in unstressed rats (DAT group x age effect $F_{(1,21)}$=17.317, p=0.0004; n=5-8 rats/group; p=0.00008). Aging also increased D_2 receptor proteins in both unstressed and PRS groups (D_2R age effect $F_{(1,20)}$=11.576, p= 0.002; n=4-8 rats/group).

The striatal synaptic machinery is indicated in **Fig. 6D**. Indeed, PRS reduced levels of syntaxin-1 at both ages (syntaxin-1 group effect $F_{(1,21)}$=8.526, p=0.008), with a more prominent effect in aged animals (adult group CONT *vs.* PRS p=0.07, aged group CONT *vs.* PRS p=0.029). Rab3a levels were also reduced in PRS rats at both ages (Rab3a group effect $F_{(1,21)}$= 5.810, p=0.02 n=5-8 rats/group) however with a significant group effect only in adult rats (CONT adult *vs*. PRS adult p= 0.044). Synaptophysin (SYP) was increased by aging in both unstressed and PRS rats (SYP age effect, $F_{(1,20)}$=21.020, p=0.001; n=5-8 rats/group). Aging enhanced SNAP-25 protein levels in both groups (SNAP-25 age effect $F_{(1,21)}$=40.515, p=0.000002, n=5-8 rats/group). Munc-18 protein levels were not affected by either aging or PRS (munc-18: $F_{(1,20)}$=0.530, p=0.474; n=5-8 rats/group; *all blots of Fig. 6 are shown in supplementary figure **S6b***).

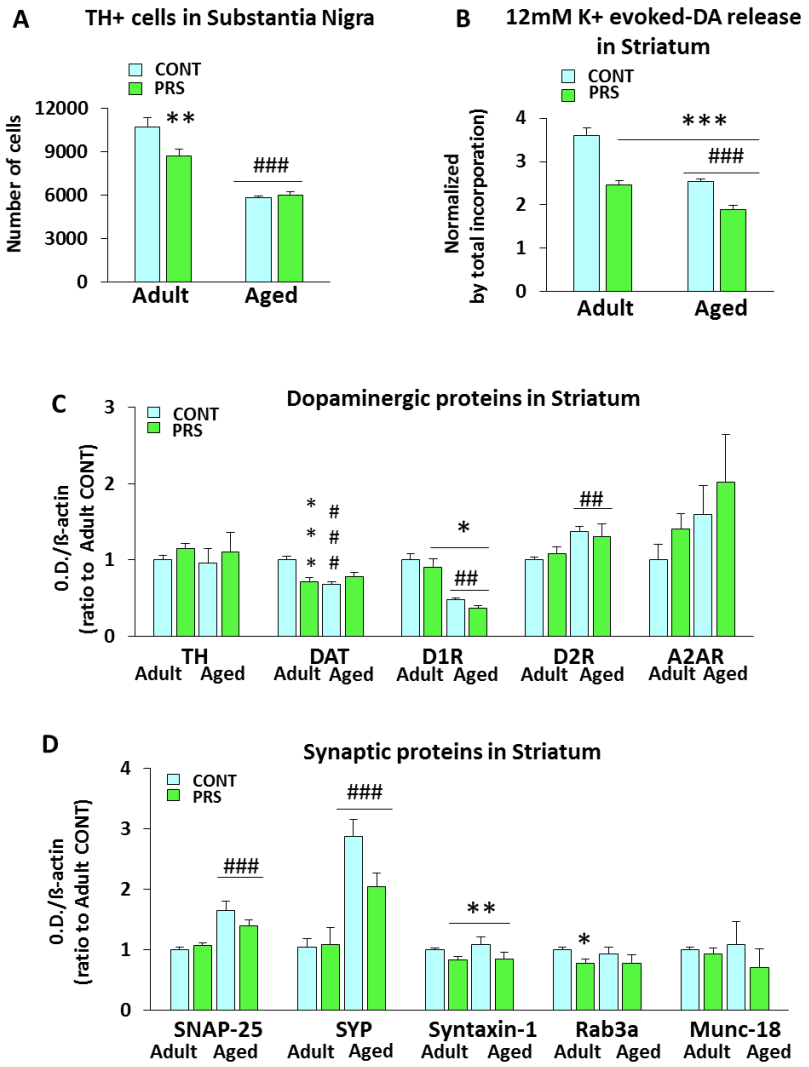

Figure 6. PRS effect nigrostriatal dopaminergic transmission during lifespan.
Here we compared adult and aged rats. In adult, PRS reduced the number of TH expressing cells with respect to age-matched control rats, while aging reduced the absolute number of TH+ cells in both groups compared to adult rats (Fig. 6A; n=7-8 rats/group). Interestingly, ^{3}H-DA release evoked by high concentrations of 12K+ in striatal synaptosomes was significantly reduced by PRS in both age groups, with comparable DA-evoked release in adult PRS rats and aged unstressed rats. Aging reduced DA release in both unstressed and PRS rats (Fig. 6B; n=5 rats/group). We then measured dopaminergic proteins in adult and aged rats (Fig. 6C). No changes were reported in the striatal levels of TH and $A_{2A}R$ (n=4-8 rats/group), while an age effect was observed in D_1R in both unstressed and PRS groups with reduced levels in comparison to adult rats (n=4-8 rats/group). Interestingly, in PRS rats, D_1R protein was lower in both age groups, with a marked effect in adult animals while DAT was already low at adulthood. Aging reduced expression of DAT protein only in unstressed rats (n=4-8 rats/group). On the other hand, aging increased D_2R protein in both unstressed and PRS groups (n=4-8 rats/group). PRS had a major impact on the striatal synaptic machinery (Fig. 6D). Indeed, PRS reduced levels of syntaxin-1 in both ages but mainly in old. Rab 3a levels were also reduce by PRS of both ages (n=5-8 rats/group) however with a significant group effect only in the adult rats. Synaptophysin (SYP) was increased by aging in both unstressed and PRS groups (n=5-8 rats/group). Aging increased levels of SNAP-25 in both groups when compared to adults. Aging and PRS affect the expression of munc-18 protein **(**n=5-8 rats/group). CONT *vs* PRS * = $p<0.05$; **= $p<0.01$; ***= $p<0.001$; adult *vs* aged ###=$p<0.001$.

DISCUSSION

Adult PRS rats displayed poor performance in a battery of behavioral tests that require a correct functioning of the striatal motor programming. Animal studies and functional imaging studies in humans suggest that the striatum is involved in instrumental conditioning (O'Doherty et al. 2004; Melief et al., 2018), and that experience of an active control over threat in the active avoidance test engage the striatum (Boeke et al., 2017). The grip strength test in rats, which measures muscular tension and rigidity, is also dependent on a correct functioning of the nigrostriatal system. In the grip strength test, lesions of the nigrostriatal system enhance the muscular force (Dunnett et al., 1998; Ma et al., 2014), and this may reflect a rat homologue of the rigidity that is a hallmark feature of DA loss in PD (Dunnett et al., 1998). Interestingly, changes induced by PRS were long lasting, and persisted in 21 months-old rats. This finding is highly relevant from a translational standpoint because age is an established risk factor for mobility decline and PD. In adult and aged PRS rats, we observed impaired behavioral performance in the pasta matrix-reaching test, which measures skilled reaching distance, direction and dexterity in rats, and in the horizontal ladder rung walking test, a task that allows the evaluation of fore- and hindlimb stepping, placing, and coordination. Of note, the lesion of the nigrostriatal system also affects these behavioral tests (Metz and Whishaw, 2002; Ballermann et al., 2001). Adult and aged PRS rats showed reduced risk-taking behavior in the open arm of the EPM. Although the EPM is not generally considered a striatum-dependent test, dopamine receptor agonists enhance the risk-taking behavior measured in this test (Drui et al., 2014). In the pasta matrix test and in the EPM, adult and aged rats behaved similarly wheras aged rats showed a poorer performance in the ladder running walking test. Interstingly, the deteriorating effect of PRS was significant in both adult and aged rats in EPM, pastax matrix and ladder rung walking test.

Neurochemical changes found in the striatum of adult PRS rats are suggestive of an impaired nigrostriatal dopaminergic transmission associated with an increased activity of the indirect pathway. The most consistent change found in both adult and aged PRS rats was a reduction of striatal DA release. In adult PRS rats, dopaminergic changes cannot be ascribed to a non-specific impairment in the release machinery, because levels of synaptic vesicle-associated proteins, except for Rab3a, and glutamate and GABA release were unchanged in adult PRS rats. In addition, in the *substantia nigra*, PRS and aging reduced the number of cells expressing TH. PRS also reduced striatal levels of DAT. Taken collectively, these findings may explain, at least in part, the reduction in DA release found in the striatum of both adult

and aged PRS rats. Surprisingly, PRS induced no changes in striatal TH protein levels in rats but increased striatal levels of DA contents and its metabolites. This could reflect a compensatory effect in response to high levels of corticosterone induced by PRS (Maccari et al., 1995), considering that corticosterone enhances catecholamine and TH levels (Busceti et al., 2019). In this regard, it has also been shown that early life stress increases corticosterone levels in the striatum (Mpofana, Daniels and Madandla, 2016). A small increase in the density of [^{3}H]-nemonapride-labeled D_2 receptors has been reported in the medial caudate/putamen of PRS rats (Adrover et al., 2007). In contrast, we found a reduction in D_1 and no changes in D_2 receptor protein levels in the striatum of adult PRS rats. The reduction in D_1 receptor expression is consistent with the poor motor performance of PRS rats because selective D_1 receptor agonists display antiparkinsonian activity (Asin et al, 1997; Martini et al., 2019). Interestingly, we found opposite changes in D_2 and A_{2A} receptor signaling (i.e., a decrease and an increase, respectively) in the striatum of adult PRS rats. D_2 and A_{2A} receptors form functional antagonistic heteromers that regulate the activity of striatopallidal neurons of the indirect pathway (Ferrè et al., 2008; Calabresi et al., 2013). For this reason, A_{2A} receptor antagonists are under clinical development for the treatment of PD (Armantero et al., 2011). It is likely that the prevalence of A_{2A} over D_2 receptor signaling in the striatum may also contribute to the impairment in striatum-dependent motor performance in PRS rats. A_{2A} receptors could be particularly sensitive to stress-related mechanisms because overexpression of A_{2A} receptors induces age-like HPA axis dysfunction by changing the activity of hippocampal glucocorticoid receptors (Batalha et al., 2016), and A_{2A} receptor blockade corrects behavioral, electrophysiological, and morphological abnormalities, and restores the activity of the HPA axis in rats subjected to maternal separation (Batalha et al., 2013). As modeled in **Figure 5**, this suggests that the hyperactivity of indirect pathway, which includes a prevalence of A_{2A} over D_2 receptor signaling, combined with the reduced dopamine release and D_2 receptor activity, as observed in adult PRS rats, can be associated with a lower performance of these animals in the behavioral tests and increase the risk for persistent and long-lasting disorders.

It was interesting to find that adult PRS rats did not show changes in striatal glutamatergic transmission and LTD at corticostriatal synapses, although striatal LTD requires a co-activation of D_1 and D_2 receptors (Calabresi et al., 1992). Early-life stress is known to induce robust changes in glutamatergic transmission in the hippocampus and prefrontal cortex (Wang et al., 2016), including deficits in the machinery controlling exocytotic glutamate release in

the ventral hippocampus (Marrocco et al., 2012, Morley-Fletcher et al., 2018). In addition, early life stress causes an impaired function of NMDA receptors and a defective long-term potentiation (LTP) of excitatory synaptic transmission in the hippocampus (Son et al., 2006). Our data suggest that the impact of PRS on glutamatergic neurotransmission and mechanisms of activity-dependent synaptic plasticity is not a general phenomenon but is strictly region-dependent.

Aging also impairs the nigrostriatal development. Indeed, in the *substantia nigra*, a reduction of the number of TH expressing cells has been shown in aged rats. In the striatum, aging reduced DAT in both groups compared to unstressed adult rats. Also, we observed a reduced effect of aging in D_1 receptors while, aging increased D_2 receptor proteins in both unstressed and PRS groups. The increased D_2 receptor protein levels during ageing could represent a compensatory mechanism aimed at mitigating the age-dependent impairment in striatal motor programming. Accumulating evidence highlight a role for synaptic vesicle-associated proteins in neurodevelopmental and neurodegenerative disorders. Functionality of the SNARE protein interactome is considered a major determinant for cognitive reserve during ageing (Ramos-Miguel et al., 2018). Aged rats showed increased striatal levels of synaptophysin and SNAP-25. SNAP-25 was found to be elevated in cerebrospinal fluid of patients with neurodegenerative disorders or mild cognitive impairment (Brinkmalm et al., 2014), suggesting that SNAP-25 is a candidate biomarker for synapse degeneration. Interestingly, SNAP-25 co-localized with D_1 receptors in cultured dopaminergic cells, and genetic deletion of SNAP-25 downregulated D_1 receptors in these cells (Yang et al., 2019). PRS reduced Rab3a protein levels in adult rats (with a trend in aged rats), and syntaxin levels in both adult and aged rats. This suggests that PRS and aging predisposes a long-term dysfunction of the release machinery in the striatum, which is relevant to the observed changes.

In conclusion, we have shown that PRS in rats causes life-long abnormalities in nigrostriatal dopaminergic transmission and reduces the performance in striatum-dependent motor tasks. In adult, a hyperactivity of the indirect pathway caused by a reduction of DA release and opposite alterations in D_2 and A_{2A} receptor signaling may lie at the core of these basal ganglia motor dysfunctions. We suggest that, PRS accelerates striatal ageing altering the developmental trajectory of the basal ganglia motor circuit. This may lower the age-dependent threshold for motor dysfunction. Dallé and Mabandla (2018) have illustrated that early life stress can induce depression and depressed patients are at risk of developing PD later in life. All together, sheds light on the nature of the motor developmental programming induced by

early life stress and indicate that early life stress is a risk factor for motor disorders in the elderly. This raises the attractive possibility that early life stress, which is poorly investigated, contributes to the understanding of the programing of human motor diseases in adulthood and aging.

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Author Contributions: SM and FN conceived and design the study; JM, RV, DB, LDM, AT, HB, GVC, VG, BP, PC, LR, FC, FB, AP, VB, GB, SMF, FN and SM acquired and analyzed the data; JM, RV, HB, GB, BP, PC, SMF, FN and SM drafted a significant portion of the manuscript or figures.

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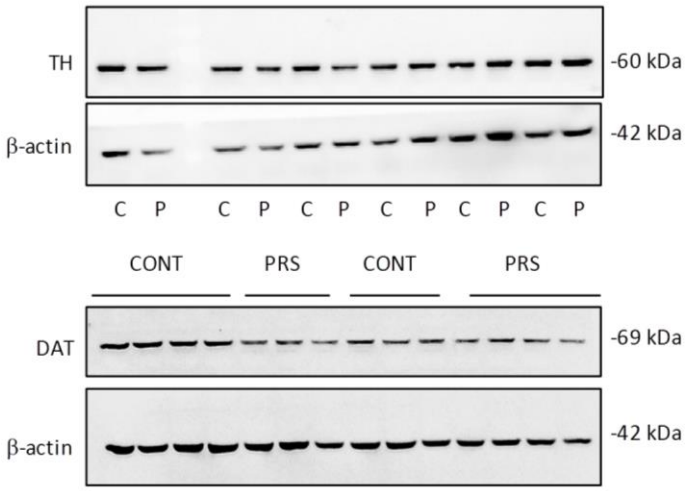
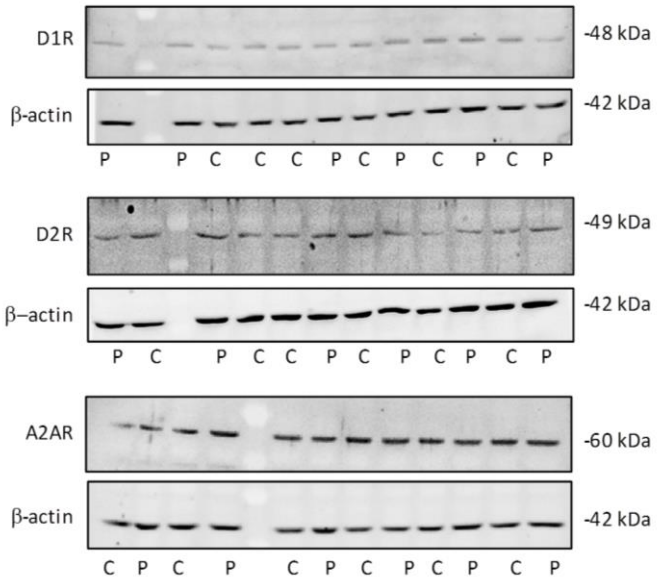
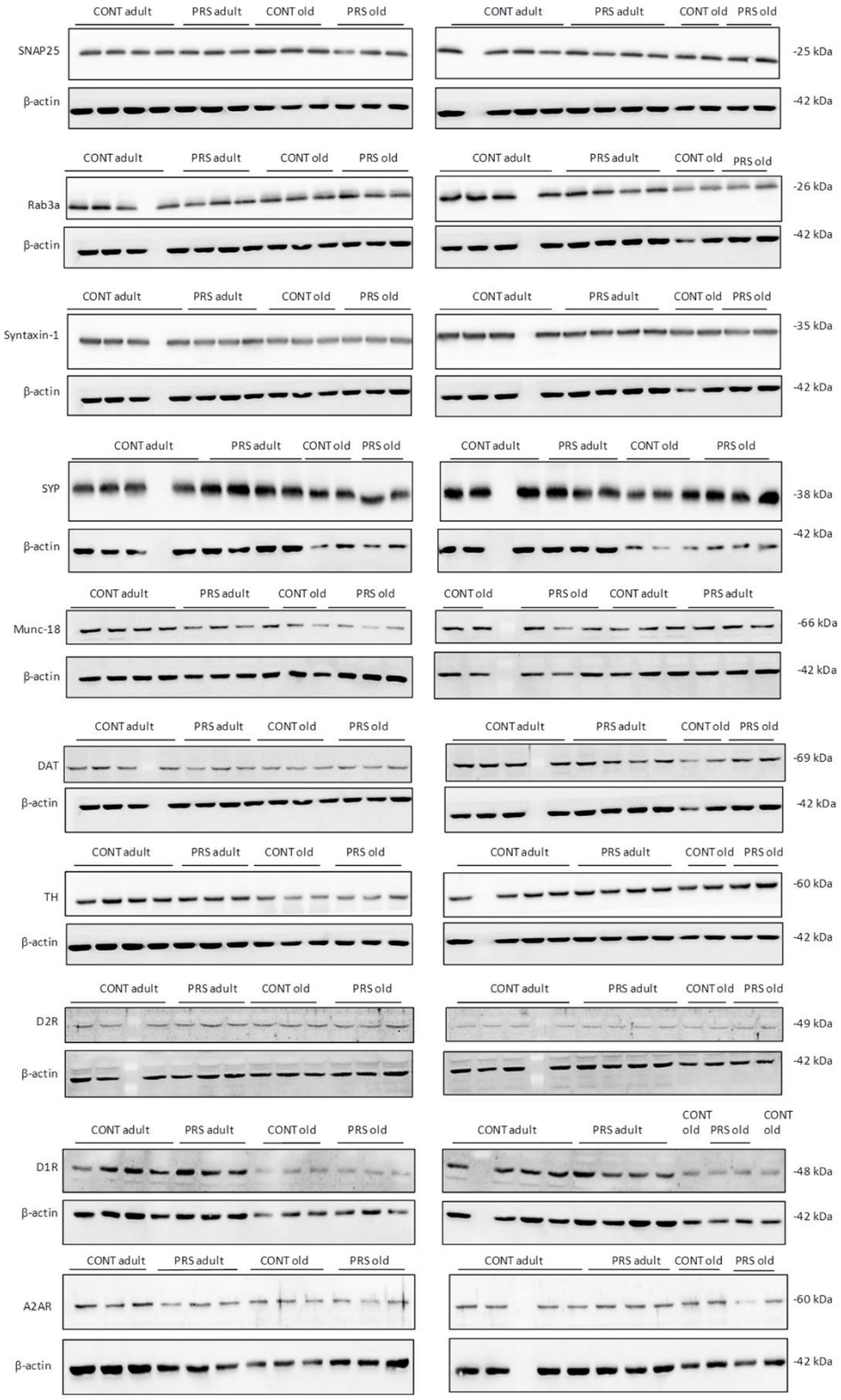
SUPPLEMENTARY DATA



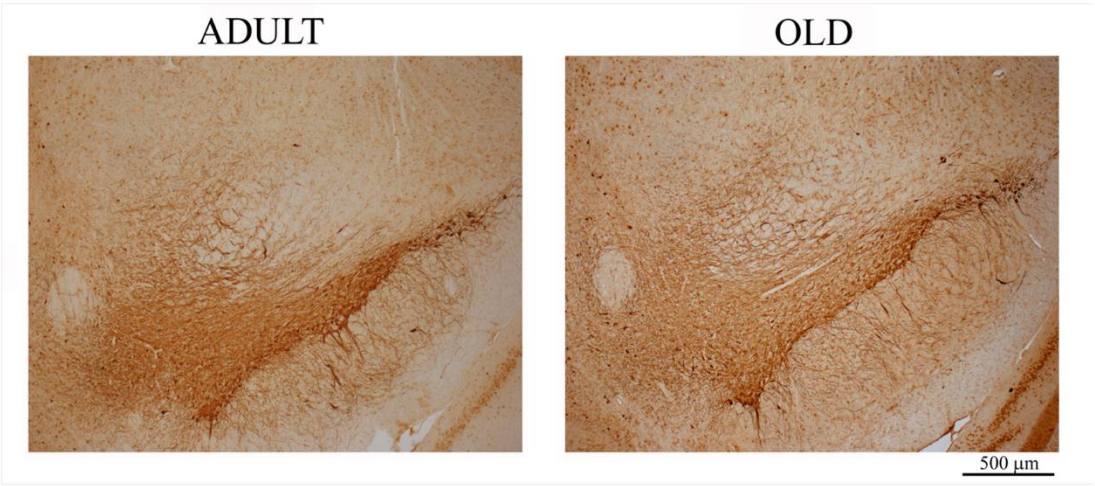
Supplementary Figure 3 (S3) Uncropped immunoblot images of TH, DAT proteins in the striatum of adult PRS (P) and control (C) male rats.



Supplementary Figure 4 (S4) Uncropped immunoblot images of D1, D2, and A_{2A} receptors in the striatum of adult PRS (P) and control (C) male rats.



Supplementary Figure 6a (S6a) Representative images of TH immunostaining in the *substantia nigra* of adult and aged rats



Supplementary Figure 6b (S6b) Uncropped immunoblot images of dopaminergic and synaptic proteins in the striatum of adult and old PRS and control (CONT) male rats.

CHAPTER 2

ROLE OF MGLU2 AND MGLU3 RECEPTORS IN BRAIN DEVELOPMENT

In **chapter one**, we observed a reduction of mGlu2/3 receptors in ventral hippocampus and prefrontal cortex in aged male and female PRS rats. The decrease of mGlu2/3 receptors induced by PRS is observed from early life (*Laloux et al., 2012*), to adult (*Zuena et al., 2008*) until aging (chapter one). Activation of mGlu2/3 receptors with the agonist, LY354740, induces an increased risk-taking behavior in male rats (*Schoepp et al., 2003*; *Linden et al., 2005*), and mGlu2/3 receptors are targets for anxiety and stress disorders in humans (*Swanson et al., 2005*). mGlu receptors are involved and are considered as drug target for neurodevelopmental disorders such as schizophrenia (*Bruno et al., 2017; Nicoletti et al., 2019*). While mGlu2 receptors have driven considerable interest compare to mGlu3 receptors, several evidences suggest that mGlu3 receptor could play a critical role in cognitive impairments. Indeed, the deletion of mGlu3 receptors in mice lead to behavioral alterations such as hyperactivity in the open field, light/dark transition, and impaired working memory and contextual memory (*Lainiola et al., 2014*; *Fujioka et al., 2014*); pharmacological treated patients with schizophrenia show an alteration in post-mortem mGlu3 receptor dimerization (*Corti et al., 2007*) and importantly, polymorphic variants of the gene encoding for metabotropic glutamate receptor 3 seem linked to schizophrenia (*Saini et al., 2017*). Furthermore, mGlu3 receptor is highly expressed in prefrontal cortex and other brain regions during the first days of postnatal development instead of mGlu2 receptor (*Catania et al., 1994*). This time window is important for GABAergic system development, particularly because of the GABA shift from excitatory to inhibitory (*Rivera et al., 1999*; *Ben Ari et al., 2007*) and the establishment of network oscillations (*Sohal et al., 2009*; *Gonzales Burgos et al., 2011*). Growing body of evidences suggest that abnormalities in GABAergic interneurons such as parvalbumin interneurons, are linked to the pathophysiology of schizophrenia (*Lodge et al., 2009*; *Metzner et al., 2019*; *Lewis et al., 2011*; *Chung et al., 2017*).

Thus, we further investigated the role of mGlu3 and mGlu2 receptors on the developmental trajectory of cortical GABAergic transmission in mice.

In this purpose, we analyzed in mGlu2$^{-/-}$ and mGlu3$^{-/-}$ mice several major events occurring early after birth that contribute to shape the developmental trajectory of inhibitory GABAergic transmission in the cerebral cortex. We used a multi-modal approach including genomic, proteomic, imaging and electrophysiological analysis.

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Developmental abnormalities in cortical GABAergic system in mice lacking mGlu3 metabotropic glutamate receptors

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ABSTRACT: Polymorphic variants of the gene encoding for metabotropic glutamate receptor 3 (mGlu3) are linked to schizophrenia. Because abnormalities of cortical GABAergic interneurons lie at the core of the pathophysiology of schizophrenia, we examined whether mGlu3 receptors influence the developmental trajectory of cortical GABAergic transmission in the postnatal life. mGlu3$^{-/-}$ mice showed robust changes in the expression of interneuron-related genes in the prefrontal cortex (PFC), including large reductions in the expression of parvalbumin (PV) and the GluN1 subunit of NMDA receptors. The number of cortical cells enwrapped by perineuronal nets was increased in mGlu3$^{-/-}$ mice, suggesting that mGlu3 receptors shape the temporal window of plasticity of PV^{+} interneurons. Electrophysiological measurements of $GABA_A$ receptor–mediated responses revealed a more depolarized reversal potential of GABA currents in the somata of PFC pyramidal neurons in mGlu3$^{-/-}$ mice at postnatal d 9 associated with a reduced expression of the K^+/Cl^- symporter. Finally, adult mGlu3$^{-/-}$ mice showed lower power in electroencephalographic rhythms at 1–45 Hz in quiet wakefulness as compared with their wild-type counterparts. These findings suggest that mGlu3 receptors have a strong impact on the development of cortical GABAergic transmission and cortical neural synchronization mechanisms corroborating the concept that genetic variants of mGlu3 receptors may predispose to psychiatric disorders.—Imbriglio, T., Verhaeghe, R., Martinello, K., Pascarelli, M. T., Chece, G., Bucci, D., Notartomaso, S., Quattromani, M., Mascio, G., Scalabrì, F., Simeone, A., Maccari, S., Del Percio, C., Wieloch, T., Fucile, S., Babiloni, C., Battaglia, G., Limatola, C., Nicoletti, F., Cannella, M. Developmental abnormalities in cortical GABAergic system in mice lacking mGlu3 metabotropic glutamate receptors. FASEB J. 33, 14204–14220 (2019). www.fasebj.org

KEY WORDS: mGlu3 receptors · cortical interneurons · development · oscillations · perineuronal nets

Metabotropic glutamate (mGlu) receptors are linked to the pathophysiology of schizophrenia and are candidate drug targets for therapeutic intervention (1, 2). mGlu receptors form a family of 8 subtypes, of which mGlu1 and mGlu5 receptors are coupled to $G_{q/11}$, whereas all other subtypes are coupled to $G_{i/o}$ (3). Selective disruption of mGlu5

ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; Arx, aristaless; bHLH, basic helix-loop-helix; CB1, cannabinoid receptor type 1; CCK, cholecystokinine; CSPG, chondroitin sulfate proteoglycan; EEG, electroencephalographic; E_{GABA}, reversal potential of GABA currents; GAD, glutamate decarboxylase; GRM3, mGlu3 receptor gene; KCC2, K^+/Cl^- symporter; LSD, least significant difference; mGlu, metabotropic glutamate; MP, membrane potential; NPY, neuropeptide Y; PAM, positive allosteric modulator; PFC, prefrontal cortex; PND, postnatal day; PNN, perineuronal net; PV, parvalbumin; RTN, reticular thalamic nucleus; Sst, somatostatin; VIP, vasoactive intestinal peptide; WFA, *Wisteria floribunda* agglutinin

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receptors in parvalbumin $(PV)^+$ GABAergic interneurons in mice induces neurophysiological and behavioral abnormalities that are reminiscent of those occurring in schizophrenia (4), and positive allosteric modulators (PAMs) of mGlu5 receptors are under development as antipsychotic agents (5).

Drugs that activate both mGlu2 and mGlu3 receptors have shown efficacy in behavioral tests that are predictive of antipsychotic activity (6), and pomaglumetad, a prodrug of the mGlu2/3 receptor agonist, LY404039, showed therapeutic efficacy in subgroups of patients affected by schizophrenia (7). The antipsychotic activity of mGlu2/3 receptor agonists appears to be mediated by mGlu2 receptors because it is abolished in mGlu2 receptor knockout mice and is shared by mGlu2 receptor PAMs (8, 9). However, the mGlu2-centric hypothesis of schizophrenia is challenged by the following observations: *1*) mGlu3 receptor knockout mice show behavioral and neurochemical abnormalities that are consistent with a psychotic-like phenotype (10, 11); *2*) alterations in mGlu3 receptor dimerization have been found in the frontal cortex of patients with schizophrenia (12); and *3*) polymorphic variants of the mGlu3 receptor gene (GRM3) are associated with schizophrenia (13, 14). A relation between mGlu3 receptors and schizophrenia is strenghtened by the finding that activation of mGlu3 receptors boosts mGlu5 receptor signaling, and mGlu3 and mGlu5 receptors interact in the induction of long-term depression in the prefrontal cortex (PFC) (15). Expression of mGlu3 receptors is widespread in the CNS. *In situ* hybridization and immunohistochemical analysis showed high levels of expression in the reticular thalamic nucleus (RTN) and moderate levels of expression in the cerebral cortex, amygdala, and other brain regions (16–18). mGlu3 receptors are present in both neurons and glial cells, and they are expressed in all components of the tripartite synapse (*i.e.*, axon terminals, dendritic spines, and surrounding astrocytes) (18). mGlu3 and mGlu5 receptors share the same developmental pattern of expression. The transcript of both receptor subtypes is highly expressed in the cerebral cortex and other brain regions in the first 7–9 d of postnatal development, and expression declines afterward. This pattern is not shared by other mGlu receptor subtypes (19).

The first 9 d of postnatal life are critical for terminal differentiation and functional specialization of cortical PV^+ GABAergic interneurons, which innervate axon segment of pyramidal neurons, and play a key role in network oscillations (20). In addition, profound changes in GABAergic transmission occur during early postnatal life, such as the excitatory-to-inhibitory GABA shift because of a sharp reduction in intracellular Cl^- concentrations (21).

Starting after the second week of postnatal life, cortical PV^+ interneurons in mice begin to be enwrapped by highly organized, lattice-like macromolecular structures of the extracellular matrix called perineuronal nets (PNNs), which are formed by a linear nonsulfated hyaluronic acid polymer joined to chondroitin sulfate proteoglycans (CSPGs) through a series of linking proteins (22–25). It has been proposed that PNNs restrain the plasticity of PV^+ interneurons, and their deposition concides with the closure of critical periods of CNS plasticity (26). In the PFC, PNNs have been shown to regulate the inhibitory currents onto pyramidal neurons, thereby influencing cortical network activity and mechanisms of neuronal plasticity (27, 28). Thus, an optimal level of PNNs within the appropriate time windows may be essential for the correct balance between excitation and inhibition underlying cognitive function.

Preclinical studies and analysis of human brain tissue suggest that abnormalities in PV^+ GABAergic interneurons are linked to the pathophysiology of schizophrenia (29). Reduced levels of neurochemical markers of PV^+ interneurons are found in the PFC of subjects with schizophrenia (30–34). PV^+ interneurons play a key role in the generation of electroencephalographic (EEG) oscillations at γ-frequency during working memory (35), and this neurophysiological mechanism may be abnormal in schizophrenia (36, 37). Changes in the density of PNNs have also been reported in the PFC and other brain regions of individuals affected by schizophrenia (34, 38–41).

We hypothesized that mGlu3 receptors may affect not only the development and functional specialization of PV^+ GABAergic interneurons but also large-scale brain neural synchronization and desynchronization mechanisms underling cortical EEG oscillations in quiet wakefulness. This hypothesis was tested by a multidisciplinary approach based on: *1*) expression analysis of selected neurochemical markers of PV^+ and other GABAergic interneurons in the PFC across postnatal development; *2*) electrophysiological analysis of GABAergic transmission in the PFC; *3*) immunohistochemical analysis of PNNs; and *4*) evaluation of cortical EEG oscillations underpinning neural synchronization and desynchronization mechanisms in quiet wakefulness. The whole analysis was performed in wild-type and mGlu3 receptor knockout ($mGlu3^{-/-}$) mice. When appropriate, $mGlu2^{-/-}$ mice were used for comparative purposes.

MATERIALS AND METHODS

Animals

Adult male and female CD1 mice were housed under controlled conditions ($T = 22°C$; humidity = 40%) on a 12-h light/dark cycle with food and water *ad libitum*. Experiments were performed following the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA) to minimize the number of animals and animal suffering. The experimental protocol was approved by the Ethical Committee of Neuromed Institute (Pozzilli, Italy) and approved as a current research project at Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Neuromed by the Italian Ministry of Health. mGlu2 and mGlu3 receptor knockout mice ($mGlu2^{-/-}$ and $mGlu3^{-/-}$ mice) with a CD1 genetic background were kindly provided by Eli Lilly and Co. (Indianapolis, IN, USA). Mouse strains were originally generated by homologous recombination (42). One male and 2 females of $mGlu2^{-/-}$, $mGlu3^{-/-}$, and wild-type CD1 mice were housed for homozygous mating. We did not use littermates generated by heterozygous mating to minimize the number of mice (experiments were performed exclusively on homozygous mice). For appropriate embryonic staging, noon of the day of vaginal plug detection was designated as embryonic day (E)0.5. E12.5 embryos were used for immunohistochemical analysis of homobox and basic helix-loop-helix (bHLH) genes. Mice at

postnatal days (PNDs) 1, 9, 21, 30, and 75 were used for biochemical analysis of interneuron-related genes, for electrophysiological analysis of GABAergic neurotransmission, and for immunohistochemical analysis of PNNs. Wild-type and mGlu3$^{-/-}$ mice at 75 PNDs were used for EEG recording.

Immunohistochemical analysis of homeobox and bHLH genes

Embryos were fixed in 4% paraformaldehyde and embedded in wax. Adjacent sections were processed for immunohistochemistry. Sections were deparaffinized in Bio-Clear, rehydrated, and incubated overnight at room temperature with primary antibodies (0.5% milk, 10% fetal bovine serum, 1% bovine serum albumin in H_2O + Na-Azide 0.02%). The following antibodies were used: Mash1 (mouse, IgG1, 556604, 1:150; BD Pharmingen, San Jose, CA, USA), Pax6 (rabbit, AB5409; 1:2000; Chemicon, Billerica, MA, USA), Nkx2.1 (rabbit, sc13040, 1:1500; Santa Cruz Biotechnology, Dallas, TX, USA), and Otx2 (rabbit, ab114138, 1:10,000; Abcam, Cambridge, United Kingdom). The MACH 4 Universal HRP-Polymer Polymer Detection Kit (M4U534; Biocare Medical, Pacheco, CA, USA) was used for immunohistochemical detection. The specificity of Mash1, Pax6, Nkx2.1, and Otx2 was validated in mouse embryos and is described in detail in the Supplemental Fig. S1*B*, *C*.

Measurements of mRNA levels of interneuron-related genes

Mice were euthanized by decapitation at PND1, 9, 30, and 75. The brains were removed and the PFC immediately dissected and frozen on liquid nitrogen. PFC was handly dissected by performing a coronal cut with a razor blade at ~1.5-mm anterior to bregma in PND30 and PND75 mice and 0.5–1-mm anterior to bregma in PND1 and PND9 mice. We have then removed the medial portion of both hemisheres in the section anterior to the coronal cut with tissue forceps with 0.5-mm tips. The dissected tissue includes the cingulate cortex, the prelimbic and infralimbic cortex, the dorsal peduncular cortex, the medial orbital cortex, and the most medial part of the secondary motor cortex. Total RNA was extracted using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The RNA was further treated with DNase (Qiagen, Germantown, MD, USA), and single strand cDNA was synthesized from 1.5 μg of total RNA using Superscript III (Thermo Fisher Scientific) and random hexamers. Real-time PCR was performed on 15 ng of cDNA by using specific primers and Power SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) on an Applied Biosystems Step-One instrument.

Thermal cycler conditions were as follows: 10 min at 95°C, 40 cycles of denaturation (15 s at 95°C), and combined annealing-extension (1 min at 58–60°C). Primer sequences of all genes are reported in Supplemental Table S1. mRNA copy number for each gene was calculated from serially diluted standard curves simultaneously amplified with the samples and normalized with respect to the transferrin receptor mRNA copy number. Each sample was analyzed in duplicate together with 2 negative controls.

Western blot analysis of PV, GluN1 subunit of NMDA receptors, and the K^+/Cl^- symporter

PV and GluN1 protein levels were examined in the PFC at PND30 and PND75. The K^+/Cl^- symporter (KCC2) protein levels were examined in the PFC at PND9 and PND30. Tissue was dissected out and homogenized at 4°C in a buffered solution composed of Tris-HCl pH 7.5, 10 mM; NaCl, 150 mM; SDS 10%, EDTA, 5 mM; PMSF, 10 mM; IGEPAL, 1%; leupeptin, 1 μg/ml; and aprotinin, 1 μg/ml. Equal amounts of proteins (20 μg) from supernatants were separated by 12% SDS polyacrylamide for PV protein and 8% for GluN1 and KCC2 proteins. After separation, proteins were transferred on immunoblot PVDF membranes. Membranes were incubated with the primary antibody and then incubated for 1 h with the secondary antibody anti-mouse or anti-rabbit (1:7000, peroxidase-coupled). The following primary antibodies were used: rabbit monoclonal anti-PV (ab11427, 1:2500; Abcam), mouse monoclonal anti-β-tubulin (sc-58886, 1:1000; Santa Cruz Biotechnology); rabbit polyclonal anti-KCC2 (ab49917, 1:2000; Abcam), rabbit polyclonal anti-GluN1 (ab109182, 1:5000; Abcam); and mouse monoclonal anti-β-actin (A5316, 1:50,000; MilliporeSigma, Burlington, MA, USA). Immunostaining was revealed by the enhanced ECL Western blotting analysis system (Hybond ECL; GE Healthcare, Waukesha, WI, USA) or by the Chemidoc computerized densitometer (Bio-Rad), and quantified by ImageLab 3.0 software (Bio-Rad).

Stereological cell counting of PV^+ neurons in the PFC

Brains from wild-type and mGlu3$^{-/-}$ mice at PND9 and PND30 were dissected out, fixed in Carnoy's solution (ethanol 60%, acetic acid 10%, and chloroform 30%), and included in paraffin. Tissue sections (20 μm) were incubated overnight with rabbit polyclonal anti-PV antibodies (ab11427, 1:100 at PND9; Abcam and PV27, 1:500 for PND30; Swant, Fribourg, Switzerland), and then for 1 h with secondary biotin-coupled anti-rabbit antibodies (1:200; Vector Laboratories, Burlingame, CA, USA). For detection, 3,3-diaminobenzidine tetrachloride (MilliporeSigma) was used. Control staining was performed without the primary antibodies. The number of PV^+ cells in the PFC (from +2.1 to +1.34 from bregma according to the Paxinos (43), for PND30 mice and the corresponding sections for PND9 mice) was assessed by stereological technique and optical fractionator using a Zeiss Axio Imager M1 microscope equipped with a motorized stage, a focus control system (Zeta axis), and a digital video camera (44). The software Image-Pro Plus 6.2 for Windows (Media Cybernetics, Rockville, MD, USA) equipped with a Macro was used for the analysis of digital images. Macro was obtained by Immagine and Computer (Milan, Italy). The characteristics of this Macro have been previously reported in Gundersen and Jensen (45). The analysis was performed on 4 sections of 20 μm, sampled every 220 μm in the rostrocaudal extension of the PFC (medial portion of the sections containing the cingulate cortex, the prelimbic cortex, the infralimbic cortex, and the dorsal peduncular cortex) using a grid of disectors with a counting frame of 40 × 40 μm, and a grid size (distance between disectors center to center) of 150 × 150 μm. The total number of PV^+ cells was computed according to the formula: $N = \Sigma(n) \times 1/SSF \times 1/ASF \times 1/TSF$, where n is the total number of cells counted on each disector; SSF (fraction of sections sampled) is the number of regularly spaced sections used for counts divided by the total number of sections across the areas; ASF (area sampling frequency) is the disector area divided by the area between disectors (disector area × disector number/region area); and TSF (thickness sampling frequency) is the disector thickness divided by the section thickness. The Cavalieri estimator method was used to evaluate the volume of the PFC (44).

Immunohistochemical analysis of Cat-315^+ PNNs and *Wisteria floribunda* agglutinin$^+$ PNNs

We measured the number of cortical cells enwrapped by PNNs stained by either the anti-CSPG antibody, Cat-315, or the lectin, *Wisteria floribunda* agglutinin (WFA), in the PFC or in 3 portions

of the cerebral cortex (Cat-315 and WFA) of wild-type and mGlu3$^{-/-}$ mice at PND21 and PND30. Free-floating brain slices were rinsed in PBS, quenched in 3% H_2O_2 and 10% methanol for 15 min, and blocked in 5% normal donkey serum and 0.25% triton X-100 in PBS for 1 h at room temperature. For Cat-315 staining, sections were incubated overnight at 4°C with monoclonal mouse anti-CSPG antibodies (Cat-315, Mab1581, 1:3000; MilliporeSigma) diluted in blocking solution. Following rinses with 2% normal donkey serum and 0.25% triton X-100 in PBS, sections were incubated with a donkey anti-mouse secondary antibody (1:400) for 90 min at room temperature for 3,3-diaminobenzidine tetrachloride staining. Bright-field pictures were acquired using an Olympus BX60 microscope. We also performed double immunofluorescent staining for Cat-315 and PV (with monoclonal rabbit anti-PV antibodies, 1:1000, PV27; Swant) using secondary donkey anti-mouse Alexa Fluor 488 antibody (1:200; Thermo Fisher Scientific) and donkey anti-rabbit Cy3 antibody (1:200; Jackson ImmunoResearch, Cambridge, United Kingdom). WFA fluorescent staining was performed using biotin-conjugate WFA (1:500, L1516; MilliporeSigma) and fluorescent secondary antibody (Streptavidin Alexa Fluor 488, 1:200; Thermo Fisher Scientific). Sections were examined with a Zeiss Carl Axiophot2 microscopy (Carl Zeiss, Oberkochen, Germany) and processed with NIS-elements F3.0.

For Cat-315 staining, bright-field cell counting was performed in 3 coronal sections per brain (+0.98, +0.02, and −1.06 mm relative to bregma) as previously reported in Quattromani *et al.* (46). In brief, composite micrographs of both hemispheres were acquired through a ×4 magnification objective using the CellSens Dimension Software (BX60; Olympus, Tokyo, Japan). An optical grid was used to define distances and draw the boundaries of the cortex in both hemispheres according to the Paxinos atlas (43). Cat-315$^+$ cells were counted in the whole cortical mantle and in all cortical layers of the 3 anatomical sections bilaterally at PND21 and unilaterally at PND30.

For WFA staining, cell counting was performed unilaterally in the same 3 coronal sections used for Cat-315 staining (+0.98, +0.02, and −1.06 mm from bregma), with the difference that cells were counted at ×10 magnification uniltaterally only in the rectangular areas highlighted in gray (and not in the whole cortical mantle). WFA$^+$ cells were also counted in 3 PFC sections at +1.78, +1.54, and +1.34 from bregma. In the latter sections, cell counting was performed at 10× magnification in the medial portion containing the prelimbic cortex, infralimbic cortex, and dorsal peduncular cortex in all sections, and cingulate cortex 2 in the +1.34 section.

Electrophysiological analysis of GABAergic transmission in the PFC

Cortical slices were prepared from PND9 and PND30 wild-type and mGlu3$^{-/-}$ mice. Coronal slices (350 μm) were cut in glycerol-based artificial cerebrospinal fluid (ACSF) with a vibratome (Leica VT 1000S; Leica Microsystems, Wetzlar, Germany), placed in a slices incubation chamber at room temperature with oxygenated ACSF, and transferred to a recording chamber within 1–6 h after slices preparation. Whole-cell patch-clamp recordings were performed on pyramidal neurons at 24–25°C. Membrane currents were recorded using glass electrodes (8–10 MΩ) filled with the following (in millimolars): 111 K-gluconate, 6 KCl, 0.5 $CaCl_2$, 10 HEPES, 5 BAPTA, 2 Mg-ATP; pH 7.3, with KOH. The calculated Cl^- reversal potential for our solution was −77 mV. Resting membrane potential (MP) was −64 ± −3 mV. GABA was photolyzed from bis(2,2′-bipyridine-*N*,*N*′)triphenylphosphine)-4-aminobutyric acid ruthenium hexafluorophosphate complex (RuBi GABA, ab120409; Abcam) using local uncaging as previously described in Khirug *et al.* (47). RuBi GABA (2.5 mM) was dissolved in ACSF and delivered to cells by pressure applications (1–5 ψ; Picospritzer II, General Valve) from glass micropipettes positioned above whole-cell voltage-clamped neurons. For local photolysis of caged RuBi GABA, a 476-nm laser flash (LASU System; Scientifica, Uckfield, United Kingdom) was delivered to the slice, through a LUM-PlanFl ×40 water-immersion objective (Olympus). The beam yielded an uncaging spot of ~10 μm in diameter that was focused either at the soma or at dendrite trees at a distance of 50 μm from the soma. The laser power (10 mW at the objective output) and flash duration (10 ms) were set at a level that provided a good signal-to-noise ratio for uncaging-evoked currents. The current/voltage (I/V) relationship was determined by varying the holding potential from −80 to −30 mV (10 mV steps, 3 s long). Peak current amplitudes were plotted against the MP to measure the local reversal potential of GABA currents (E_{GABA}). The laser flash evoked no responses in the absence of RuBi GABA. Junction potential calculated for our solution was −14 mV, and MP was corrected accordingly.

Video-EEG data recording and analysis

EEG recording and analysis of data were performed in 9 mGlu3$^{-/-}$ and 9 wild-type mice. These mice were adapted to the laboratory conditions for 1–2 wk before implantation of EEG epidural electrodes. During this period, they were housed under standard conditions (see above) and received a gentle handling for about 5–10 min per day. Food and water were available *ad libitum*. Stainless steel insulated electrodes with mounting screw and socket contacts (3.2-mm screw wire length of 10 mm, E363/20/SPC; Bilaney, Kent, United Kingdom) were implanted under isoflurane (3%) anesthesia and intraperitoneal injection of rompun (20 mg/ml) at the dose of 75 mg/kg + zoletil (1 mg/ml) at the dose of 20 mg/kg. Mice were placed in a stereotaxic head frame where the exploring EEG electrodes were positioned on the *dura mater* surface overlying the anterior frontal and posterior parietal cortical regions using the following stereotaxic coordinates: AP +2.8 mm and ML at −0.5 mm from bregma for the frontal electrode; AP −2.0 mm and ML −2.0 mm for the parietal electrode, according to the Paxinos atlas (43). The exploring electrodes were referred to a reference electrode placed into the cerebellum, whereas the ground electrode was located in the nose bone. This electrode montage was successfully used for the investigation of the ongoing EEG oscillatory activities in wild-type and transgenic mice accumulating amyloid in the brain mice in the FP7 Innovative Medicine Initiative (IMI) of the European Project with the short name "PharmaCog" (48, 49). After the surgical procedure, mice were kept at a warm temperature to avoid anesthesia-induced hypothermia. A few hours after surgery, the mice were treated by systemic analgesics and antibiotics. In the week following electrode implantation, all mice experienced a period of full recovery without handling or EEG recordings. Afterward, mice received a gentle handling for about 5–10 min a day. EEG recordings started 10 d after surgery.

In any EEG experimental day, Grass Technologies 2011 (Twin software 4.5.3.23) were used for the simultaneous EEG and video digital recordings in a couple of mice (1 wild-type and 1 mGlu3$^{-/-}$ mouse). These mice were simultaneously connected to a system consisting of an AS40 Amplifier by a flexible cable. EEG data were recorded with a sampling rate of 200 Hz and an antialiasing bandpass filter (0.1–70 Hz). Nine couples of mice were used for a continuous video-EEG recording of 24 h (*i.e.*, 12 h with light on, and 12 h with light off). Any couple was formed by 1 wild-type and 1 mGlu3$^{-/-}$ mouse, each animal being free to move in a separate cage. The simultaneity of the video-EEG recording paired the effects of any environmental source of electric fields on EEG recording in a given couple of mice.

EEG recordings always started after the second hour from the beginning of light and darkness. EEG recordings failed in the wild-type mouse of the first recording day (*i.e.*, wild-type 01) and in the couple of mice of the last day (*i.e.*, wild-type 05 and

of the cerebral cortex (Cat-315 and WFA) of wild-type and mGlu3$^{-/-}$ mice at PND21 and PND30. Free-floating brain slices were rinsed in PBS, quenched in 3% H_2O_2 and 10% methanol for 15 min, and blocked in 5% normal donkey serum and 0.25% triton X-100 in PBS for 1 h at room temperature. For Cat-315 staining, sections were incubated overnight at 4°C with monoclonal mouse anti-CSPG antibodies (Cat-315, Mab1581, 1:3000; MilliporeSigma) diluted in blocking solution. Following rinses with 2% normal donkey serum and 0.25% triton X-100 in PBS, sections were incubated with a donkey anti-mouse secondary antibody (1:400) for 90 min at room temperature for 3,3-diaminobenzidine tetrachloride staining. Bright-field pictures were acquired using an Olympus BX60 microscope. We also performed double immunofluorescent staining for Cat-315 and PV (with monoclonal rabbit anti-PV antibodies, 1:1000, PV27; Swant) using secondary donkey anti-mouse Alexa Fluor 488 antibody (1:200; Thermo Fisher Scientific) and donkey anti-rabbit Cy3 antibody (1:200; Jackson ImmunoResearch, Cambridge, United Kingdom). WFA fluorescent staining was performed using biotin-conjugate WFA (1:500, L1516; MilliporeSigma) and fluorescent secondary antibody (Streptavidin Alexa Fluor 488, 1:200; Thermo Fisher Scientific). Sections were examined with a Zeiss Carl Axiophot2 microscopy (Carl Zeiss, Oberkochen, Germany) and processed with NIS-elements F3.0.

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EEG recordings always started after the second hour from the beginning of light and darkness. EEG recordings failed in the wild-type mouse of the first recording day (*i.e.*, wild-type 01) and in the couple of mice of the last day (*i.e.*, wild-type 05 and

mGlu3$^{-/-}$ 05) because of technical failures (*e.g.*, bad contacts of the exploring electrodes, unexpected crashes of EEG recording system during long periods of 12 h with light off). The video recordings were used for behavioral analysis according to the procedures developed in the PharmaCog project (43, 44). In the analysis of behavioral data, we classified animal behavior during ongoing EEG recordings. Two trained experimenters (C.D.P. and M.T.P.) classified recording epochs lasting 10 s into the following behavioral classes:

1. Active behavior (condition): This kind of epoch showed animals performing overt exploratory movements in the cage for most of the given period of 10 s. The exploratory movements had to be characterized by ample displacements of body parts such as trunk, head, or forelimbs, or any combination thereof. They are not to be confounded with instinctual activities.
2. Passive behavior in wakefulness (condition): This kind of epoch showed mice exhibiting passive behavior corresponding to a substantial animal immobility for most of the given period of 10 s. To avoid a misclassification with respect to the sleep, this condition had to be characterized by short immobility or small movements of the trunk, head, or forelimbs, or any combination thereof. For the same reason, the experimenter did not score as passive behavior epochs showing mice staying continuously still for 20 s (or longer).
3. Behavioral sleep state: This kind of epoch showed mice exhibiting a sleep state from a behavioral point of view. This state corresponded to an animal immobility for a long observation period lasting several minutes. Another important sign was neck muscle relaxation when detectable in the video recordings. Particular attention was again devoted to minimizing the misinterpretation between sleep state and passive conditions.
4. Instinctual behavior: This kind of epoch showed mice exhibiting instinctual activities, such as drinking, cleaning, eating, *etc.*, for most of the given period of 10 s. Special attention was paid not to include these epochs into the class denoting active behavior.
5. Undefined: This kind of epoch was characterized by a lack of clarity about the behavioral condition of the animal. Such epochs were rejected from the EEG data analysis.

For the analysis of EEG data, an EEG recording interval of 1 h was selected in the light-off condition (from 6:00 PM to 6:00 AM), corresponding to the wakefulness of the mice. The selected period had the schedule of 11:00 to 12:00 PM. Of note, this period was very far the lighting change (transition from light on to light off) to minimize effects of the stress on EEG readouts.

To avoid the confounding effects of major motor activity and sleep (we could not determine sleep stages without electro-oculographic and electromyographic recordings), the EEG data analysis was only focused on behavioral epochs (lasting 10 s) related to the passive state in the wakefulness (*i.e.*, 11:00–12:00 PM). Any epoch (lasting 10 s) of the passive state was segmented into 5 consecutive EEG epochs lasting 2 s. In the periods of interest, the EEG epochs were visually inspected to identify and reject those with EEG signals contaminated by instrumental or biologic artifacts. Afterward, a standard spectral analysis of artifact-free EEG signals was performed by digital fast Fourier transform–based procedures. The analysis was focused on the spectralrange with greatest EEG power density (*i.e.*, 2–45 Hz).

For each mGlu3$^{-/-}$ or wild-type mouse, the 2-s EEG epochs were used as an input to a fast Fourier transform–based spectral analysis using Welch technique, Hanning windowing function, and no phase shift. The EEG power density was computed with 0.5 Hz of frequency resolution (script in MatLab 6.5; MathWorks, Natick, MA, USA). The relative solutions were averaged across the following frequency bands of interest that were selected: 2–7 Hz (δ-θ), 8–13 Hz (α), 14–34 Hz (β), 35–45 Hz (γ). The same procedure was followed for wild-type mice. Logarithmic transformation was applied to reduce intervariability of EEG power density values across mice. At the end of the procedure, enough artifact-free EEG epochs related to passive wake were obtained in 7 wild-type and 8 mGlu3$^{-/-}$ mice. Data analysis of EEG power density values was performed in those mice.

Finally, we performed a control analysis to test the hypothesis that in wild-type mice, the general EEG spectral features in the period of interest (*i.e.*, 11:00–12:00 PM) did not differ from those of other periods of interest in the light off condition (from 6:00 PM to 6:00 AM), which are mainly characterized by wakefulness. To this aim, we considered 2 additional control periods, namely from 8:00 to 9:00 PM (P1) and from 9:00 to 10:00 PM (P2). In this line, we named the period of interest from 10:00 to 11:00 PM as P3. In a first step of the analysis, we selected the wild-type mice having enough artifact-free EEG epochs related to passive wake in the 3 periods of interest ($n = 7$). In a second step, the EEG power density of the artifact-free 2-s EEG epochs were compared among P1, P2, and P3 in the mentioned frequency bands (*i.e.*, 2–7, 8–13, 14–34, and 35–45 Hz).

Statistical analysis

Statistical analysis of biochemical data, immunohistochemical data, and electrophysiological data of GABAergic transmission were carried out by either Student's *t* test or 1-way ANOVA followed by Fisher's least significant difference (LSD). Statistical analysis of EEG data was performed by Statistica 10.0 packages (StatSoft, Tulsa, OK, USA; *www.statsoft.com*). An ANOVA ($P < 0.05$) used EEG power density as a dependent variable. ANOVA factors were the 2 genotypes (wild-type and mGlu3$^{-/-}$ mice) and the frequency bands (2–7, 8–13, 14–34, and 35–45 Hz). The Duncan test was used as *post hoc* test for planned comparisons. Finally, an ANOVA ($P < 0.05$) evaluated the control hypothesis that in the wakefulness day period, EEG oscillations did not differ as general spectral features in 3 distinct periods lasting 1 h (*i.e.*, P1, P2, and P3) in the wild-type group. The ANOVA-dependent variable for the analysis was the mean of the EEG power density in the frontal-parietal electrode montage. ANOVA factors were as follows: time (P1, P2, and P3) and frequency bands (2–7, 8–13, 14–34, and 35–45 Hz). The Duncan test was used for *post hoc* comparisons.

RESULTS

Genetic deletion of mGlu3 receptors lowers the expression of biochemical markers of cortical interneurons during postnatal development

We first examined whether the expression of genes that determine the fate of GABAergic interneurons in the embryonic life was influenced by genetic deletion of either mGlu2 or mGlu3 receptors. Mash1 is a bHLH transcription factor that is expressed in proliferative zones of the forebrain and is required for neuronal differentiation (50–52). Otx2 has a key role in the specification of thalamic GABAergic interneurons (53) and in the postnatal maturation of PV^{+} cortical interneurons (54–57). Pax6 is a paired-box transcription factor, which is expressed by progenitor cells in the ventricular zone of the dorsal telencephalon and regulates the number and migration of

GABAergic interneurons in the embryonic life (58, 59). The transcription factor, Nkx2.1, which is expressed in the medial ganglionic eminence of the telencephalon, has a key role in specification of GABAergic interneurons (60–63). Immunostainig performed in embryos at E12.5 did not show apparent changes in the expression pattern of Pax6, Otx2, Mash1, and Nkx2.1 in either $mGlu2^{-/-}$ or $mGlu3^{-/-}$ mice (Supplemental Fig. S1*A*).

We next examined whether the lack of mGlu2 or mGlu3 receptors could affect the expression of genes encoding for biochemical markers of GABAergic interneurons in the PFC in the first month of postnatal life. At PND1, both $mGlu2^{-/-}$ and $mGlu3^{-/-}$ mice showed a reduction in the transcripts encoding for somatostatin (Sst), vasoactive intestinal peptide (VIP), and the α_5 subunit of $GABA_A$ receptors. $mGlu3^{-/-}$ differed from $mGlu2^{-/-}$ mice in the expression of the transcripts encoding for the transcription factor aristaless (Arx), α5 subunit of GABAA receptors, and neuropeptide Y (NPY), and Reelin. Arx and CB1 mRNA levels were selectively reduced in $mGlu3^{-/-}$ mice and NPY mRNA levels were significantly reduced in $mGlu2^{-/-}$ mice and increased in $mGlu3^{-/-}$ mice, whereas Reelin mRNA levels were selectively increased in $mGlu3^{-/-}$ mice as compared with wild-type mice (**Fig. 1*A***). PV mRNA levels were undetectable at PND1.

At PND9, PV mRNA levels could be detected in PFC and were differentially regulated by mGlu2 and mGlu3 receptors. PV mRNA levels were substantially reduced in $mGlu3^{-/-}$ mice and increased in $mGlu2^{-/-}$ mice (Fig. 1*A*). The transcripts encoding for GluN1 and NPY were reduced in $mGlu3^{-/-}$ mice. CB1 receptor and $GABA_A$ δ subunit mRNA levels were reduced in both $mGlu2^{-/-}$ and $mGlu3^{-/-}$ mice (Fig. 1*A*).

At PND30, only a few transcripts were affected by the lack of mGlu2 or mGlu3 receptors. Interestingly, $mGlu3^{-/-}$ mice showed a robust reduction in PV mRNA levels, a reduction in GluN1 subunit, and an increase in carletinin and Reelin mRNA levels. $mGlu2^{-/-}$ mice only showed a reduction in PV mRNA levels (Fig. 1*A*).

We also measured PV and GluN1 protein levels at PND30 (PV protein levels were undetectable by Western blot analysis at PND9). A significant reduction in PV and GluN1 protein levels was found in $mGlu3^{-/-}$ mice at PND30 (Fig. 1*B*).

In a separate set of experiments, we extended the analysis of selected transcripts and PV and GluN1 protein levels to adult mice (PND75). The analysis was exclusively carried out in $mGlu3^{-/-}$ mice and their wild-type counterparts. As observed at PND30, adult $mGlu3^{-/-}$ mice showed a significant reduction in PV and GluN1 protein levels in the PFC. However, mRNA levels of PV, GluN1, Sst, glutamate decarboxylase (Gad)-67 (Gad1), Gad-65 (Gad2), and Reelin did not differ between $mGlu3^{-/-}$ and wild-type mice at PND75 (Fig. 2*A*, *B*).

These findings suggest that the lack of mGlu3 receptors causes an abnormal development of PV^+ GABAergic interneurons in PFC. To exclude that the observed biochemical changes could reflect alterations in the absolute number of interneurons, we also performed a stereological counting of PV^+ neurons in the PFC at PND9 and PND30. The absolute number of PV^+ interneurons did not differ between $mGlu3^{-/-}$ mice and their wild-type counterparts at both ages (Supplemental Fig. S2*A–C*).

Changes in Cat-315$^+$ and WFA$^+$ PNNs in the cortex of mGlu3$^{-/-}$ mice

We also examined whether the lack of mGlu3 receptors could affect the density of Cat-315^+ and WFA^+ PNNs at PND21 and PND30. In a first set of experiments, we measured the density of PNNs labeled by the CSPG Cat-315 antibody in $mGlu3^{-/-}$ mice and their wild-type counterparts at PND21 and PND30. Cat-315 immunostaining was faint in the PFC, and, therefore, we carried out the analysis in the cerebral cortex at 3 levels corresponding to +0.98, +0.02, and −1.06 mm from bregma. These portions of the cortex are posterior to the PFC and contain the primary and secondary motor cortex and different subregions of the somatosensory cortex in addition to area 1 and area 2 of the cingulate cortex (+0.98 and +0.02 mm from bregma) and the retrosplenia cortex (−1.06 mm from bregma). Double immunofluorescence showed that most of the neurons enwrapped by Cat-315^+ PNNs at PND21 were PV^+ (**Fig. 3*A***). An increased density of Cat-315^+ PNNs was found in the cerebral cortex of $mGlu3^{-/-}$ mice at PND21, but not at PND30 (Fig. 3*A–C*). For the evaluation of PNNs in the PFC, we performed staining for the lectin WFA, which labels *N*-acetylgalactosamines-β1 present in PNNs (64). The density of WFA^+ PNNs was also significantly increased in the PFC at PND21, but not at PND30 (**Fig. 4*A–D***). We could also confirm the increased density of PNNs in the 3 posterior regions of the cerebral cortex by WFA staining (Fig. 4*E–H*).

Cortical pyramidal neurons from PND9 mGlu3$^{-/-}$ receptor mice exhibit depolarized E_{GABA}

The shift of $GABA_A$ receptor function from excitatory to inhibitory in the early postnatal life represents a key event in the developmental trajectory of the CNS. To examine whether the lack of mGlu3 receptors could affect this process, we evaluated E_{GABA} in L5 pyramidal neurons (exhibiting the usual firing profile; **Fig. 5*A***) of PFC from wild-type and $mGlu3^{-/-}$ mice, in both somata and apical dendrites, by means of uncaging GABA experiments (47, 65). In PND9 wild-type mice, laser flashes applied to the soma (Fig. 5*B*) evoked GABA currents (I_{GABA}) in whole-cell I_{GABA} with an E_{GABA} of −78 ± 1 mV (Fig. 5*C*; $n = 11$; in agreement with the calculated E_{Cl}, −77 mV); in PND9 $mGlu3^{-/-}$ mice the E_{GABA} values were −74 ± 1 mV (Fig. 5*D, E*; $n = 10$; $P = 0.033$), indicating a less efficient Cl^- extrusion in $mGlu3^{-/-}$ mice at this age. No difference was found between E_{GABA} values measured in the apical dendrites of the same cells: −74 ± 2 and −75 ± 1 mV for wild-type and $mGlu3^{-/-}$ mice, respectively (Fig. 5*E*). Because expression of the K^+-Cl^- antiporter, KCC2, drives developmental changes in Cl^- homeostasis and E_{GABA} in neurons (66), we also examined KCC2 protein levels by immunoblotting in the PFC of wild-type and $mGlu3^{-/-}$ mice. Interestingly, KCC2

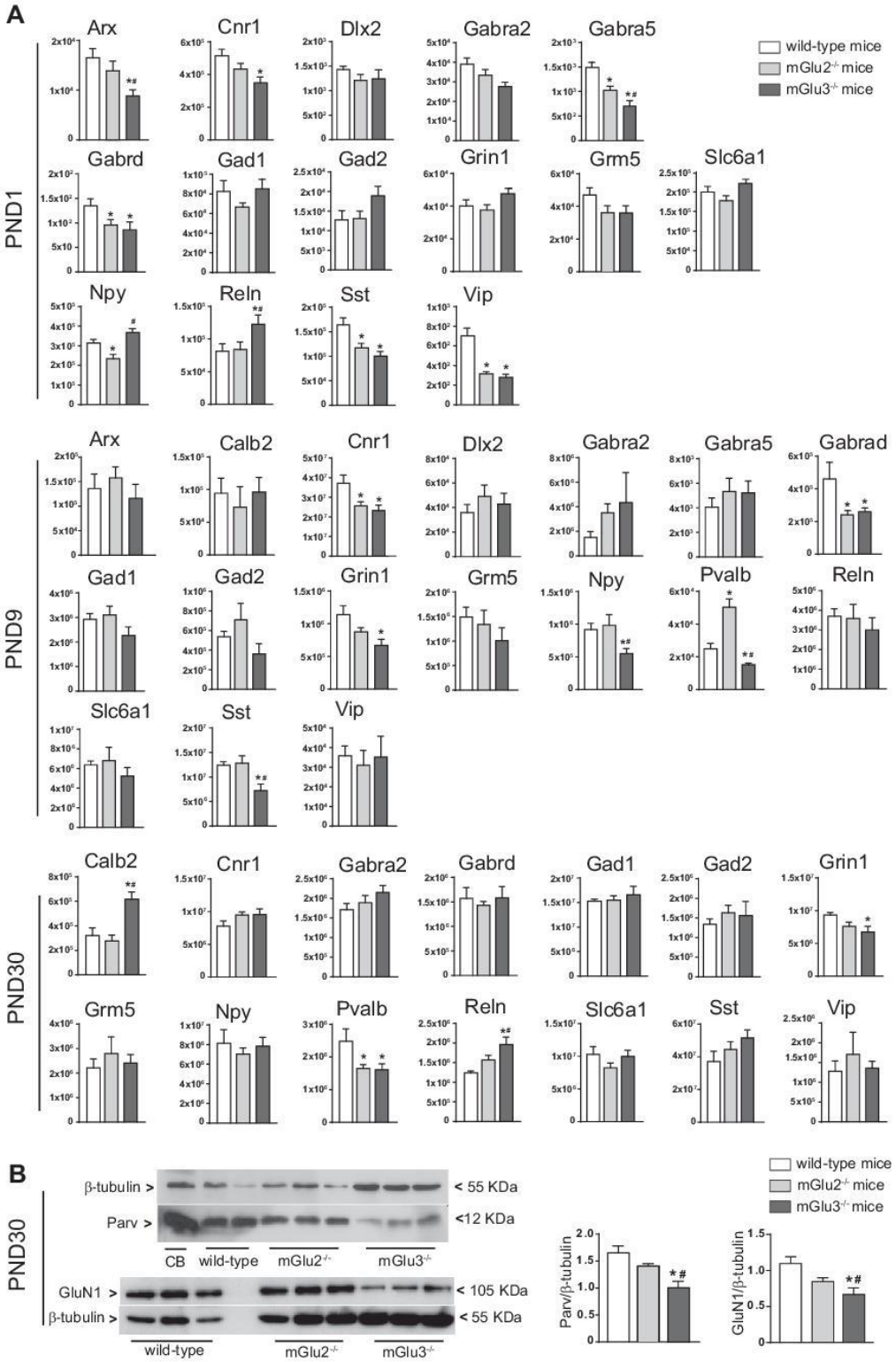

Figure 1. Influence of genetic deletion of mGlu2 or mGlu3 receptors on biochemical markers of GABAergic interneurons in the PFC during postnatal development. *A*) Changes in the transcript of interneuron-related genes at PND1, -9, and -30. Gene nomenclature is used in the figure. mRNA values, expressed as copy number, were normalized to transferrin receptor (TFRC)

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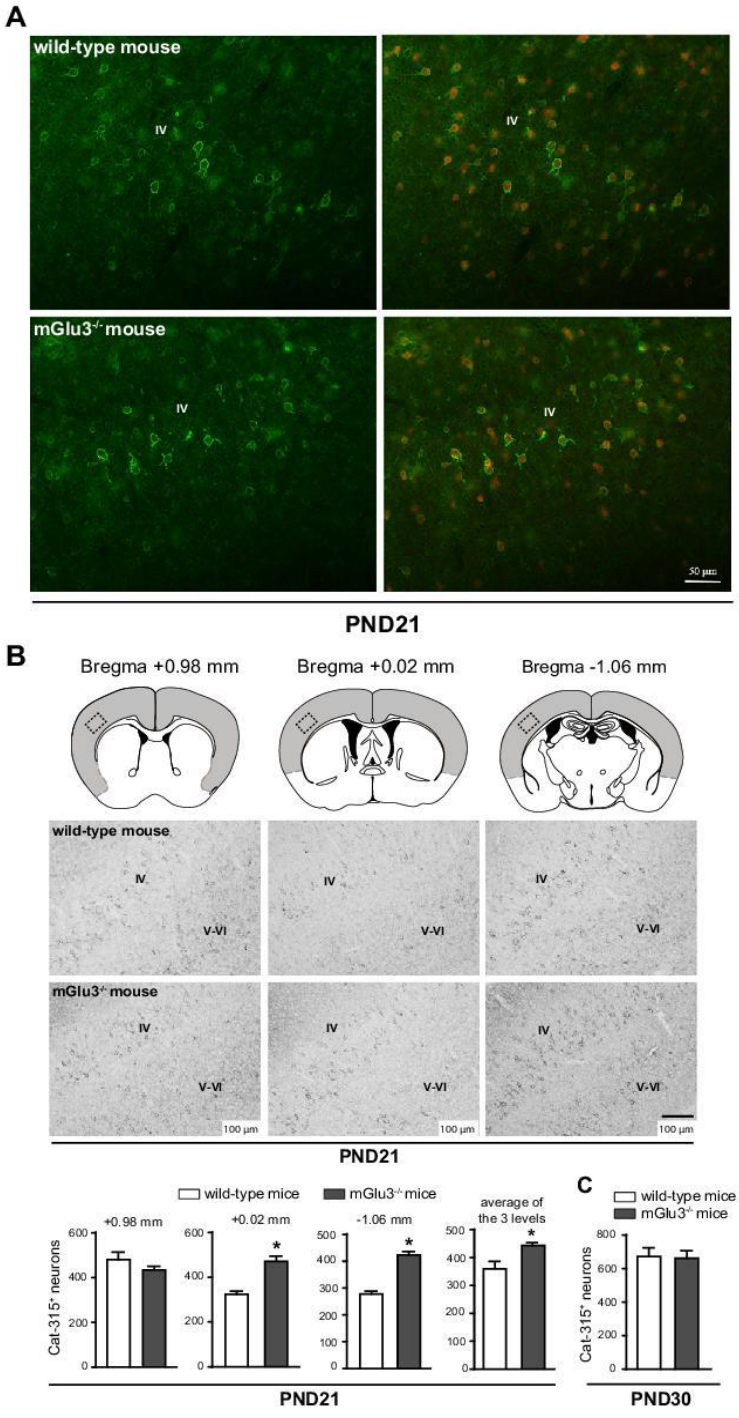

Figure 3. Increased density of Cat-315$^+$ PNNs in the cerebral cortex of mice lacking mGlu3 receptors at PND21. *A*) Double fluorescent staining for Cat-315 (green) and PV (red) in the cerebral cortex of wild-type and mGlu3$^{-/-}$ mice (at +0.02 mm from bregma). Note that the majority of neurons surrounded by Cat-315$^+$ PNNs are PV$^+$. *B*) The number of Cat-315$^+$ cells was determined in the cerebral cortex at different distances from bregma (+0.98, +0.02, and −1.06 mm). Cat-315$^+$ cells were counted bilaterally in the whole cortical mantle highlighted in gray and in all cortical layers in the 3 sections. Representative images of Cat-315 immunostaining (*B*) refer to the 3 portions of the somatosensory cortex included in the dashed rectangles. Cortical layers are indicated (*A*, *B*). Counts of Cat-315$^+$ cells in each anatomic section and average counts in the 3 sections are also shown (*B*). Values are means ± SEM of 5 mice/group *Statistically significant (Student's *t* test) *vs.* the respective values obtained in wild-type mice. +0.02 mm from bregma: $t_8 = -6.27$; $P = 0.00024$; −1.06 mm from bregma: $t_8 = -9.7$; $P = 0.00001$; average number of the 3 anatomic levels: $t_8 = 6.54$; $P = 0.0002$. *C*) Average number of neurons counted in cerebral cortex at +0.98, +0.02, and −1.06 mm from bregma in wild-type and mGlu3$^{-/-}$ mice at PND30. Cat-315$^+$ cells were counted only in 1 hemisphere. Values are means ± SEM of 3 mice/group.

density is shown at the frequency bands of 2–7, 8–13, 14–34, and 35–45 Hz. Interestingly, mGlu3$^{-/-}$ mice showed a substantial reduction in the magnitude of EEG power densities across all frequency bands as compared with wild-type mice (Fig. 6*B*). ANOVA revealed a 2-way interaction between genotypes and frequency bands ($F = 10.6$; $P < 0.01$). The planned Duncan *post hoc* analysis showed a significant difference in the magnitude of frontal-parietal EEG power density between mGlu3$^{-/-}$ and wild-type mice.

DISCUSSION

At least 4 major events occurring early after birth contribute to shape the developmental trajectory of inhibitory

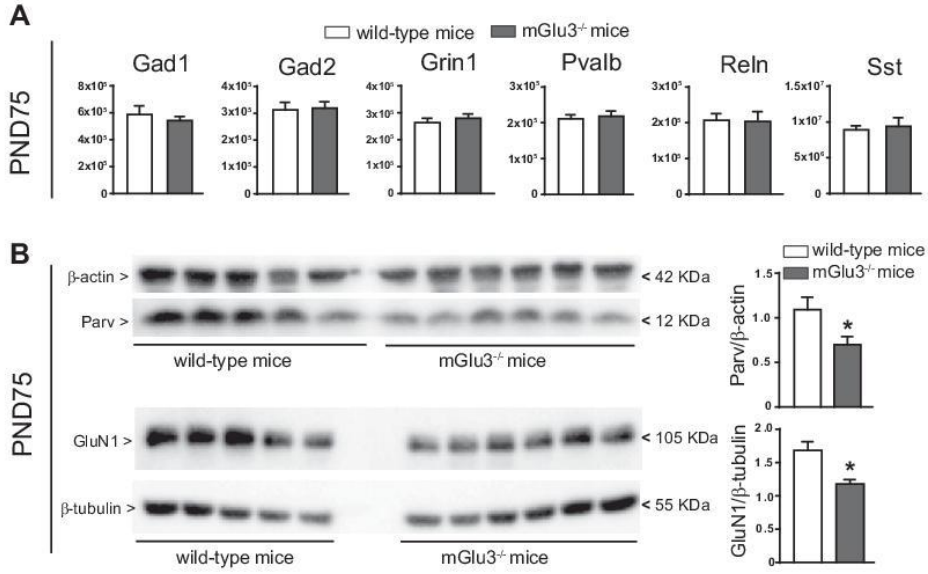

Figure 2. Analysis of selected transcripts and protein levels of interneuron-related genes in wild-type and mGlu3$^{-/-}$ mice at PND75. *A*) mRNA levels of PV, GluN1 subunit of NMDA receptors (Grin1), Sst, Gad1, Gad2, and Reelin (Reln) genes (see Fig. 1 for the corresponding proteins); *B*) Immunoblot analysis of PV and GluN1. Values are means ± SEM of 5–6 mice/group. *Statistically significant (Student's *t* test) *vs.* wild-type mice. Parv: $t_9 = 2.69$; $P = 0.0248$; GluN1: $t_9 = 4.104$; $P = 0.00266$.

protein levels were significantly reduced in mGlu3$^{-/-}$ mice (Fig. 5*F*), which is in close agreement with the more depolarized E_{GABA} found in these animals.

L5 pyramidal neurons from mGlu3$^{-/-}$ mice at PND30 showed less depolarized dendritic E_{GABA}

At PND30, somatic E_{GABA} in wild-type mice was similar to that recorded at PND9 (-76 ± 1 *vs.* -78 ± 1 mV $P = 0.374$). This was not unexpected because most of the developmental increase in KCC2 expression in the mouse cerebral cortex occurs in the first 2 wk of postnatal life (67, 68). E_{GABA} values did not differ between wild-type and mGlu3$^{-/-}$ mice at PND30 (-76 ± 1 mV and -77 ± 1 mV, $n = 9$ and $n = 10$, respectively; Fig. 5*G*; $P = 0.365$), indicating a recovery of Cl^- extrusion in mGlu3$^{-/-}$ mice after weaning. At this age, however, E_{GABA} measured in dendrites was significantly more negative in mGlu3$^{-/-}$ mice than in their wild-type counterparts (-77 ± 1 *vs.* -73 ± 1 mV, respectively; Fig. 5*H, I*; $P = 0.036$), suggesting a lower subcellular heterogeneity of Cl^- transport in mGlu3$^{-/-}$ L5 pyramidal neurons. In apparent contrast with data obtained at PND9, mGlu3$^{-/-}$ mice at PND30 showed a substantial increase in KCC2 protein levels in the PFC (Fig. 5*J*).

Influence of mGlu3 receptors on EEG oscillatory activity in adult mice

Figure 6*A* shows frontal-parietal EEG power density values in wild-type mice in 3 time intervals lasting 1 h (P1: 8:00–9:00 PM; P2: 9:00–10:00 PM; P3: 11:00–12:00 PM) during the light-off period (from 6:00 PM to 6:00 AM). Mice were mainly awake during these time intervals. All artifact-free 2-s EEG epochs were considered. The frequency bands of interest were 2–7, 8–13, 14–34, and 35–45 Hz. Of note, background levels of frontal-parietal EEG power density across all frequency bands did not differ among P3 (the time interval used for the comparative analysis between wild-type and mGlu3$^{-/-}$ mice), P1, and P2. ANOVA confirmed the lack of difference and the general reliability of the EEG spectral features in our experimental design.

Figure 6*B* shows the EEG power density computed from the bipolar frontal-parietal electrode montage in adult wild-type and mGlu3$^{-/-}$ mice. Input data from the spectral analysis referred to the animal passive behavioral state observed in the quiet wakefulness. The EEG power

and are means ± SEM of 5–9 mice/group. Statistical analysis was performed by 1-way ANOVA + LSD. Statically significant *vs.* the corresponding values of wild-type (WT) (∗) or mGlu2$^{-/-}$ (#) mice are reported in Supplemental Table S2. *B*) Western blot analysis of PV and the GluN1 subunit of NMDA receptors in the PFC at PND30. Densitometric values are means ± SEM of 6–10 mice per group. Representative blots are shown. Calb2, carletinin; CB, cerebellum from adult mice, used as positive control for Parv expression; Cnr1, CB1 receptor; Gabra2, Gabra5, and Gabrd, α_2, α_5, and δ subunits of $GABA_A$ receptors; Grin1, GluN1 subunit of NMDA receptors; Grm5, mGlu5 receptor; Reln, Reelin; Slc6a1, GAT-1. 1-way ANOVA + Fisher's LSD. $P < 0.05$ *vs.* the corresponding values of wild-type (∗) or mGlu2$^{-/-}$ (#) mice. *F* and *P* values are reported in Supplemental Table S2.

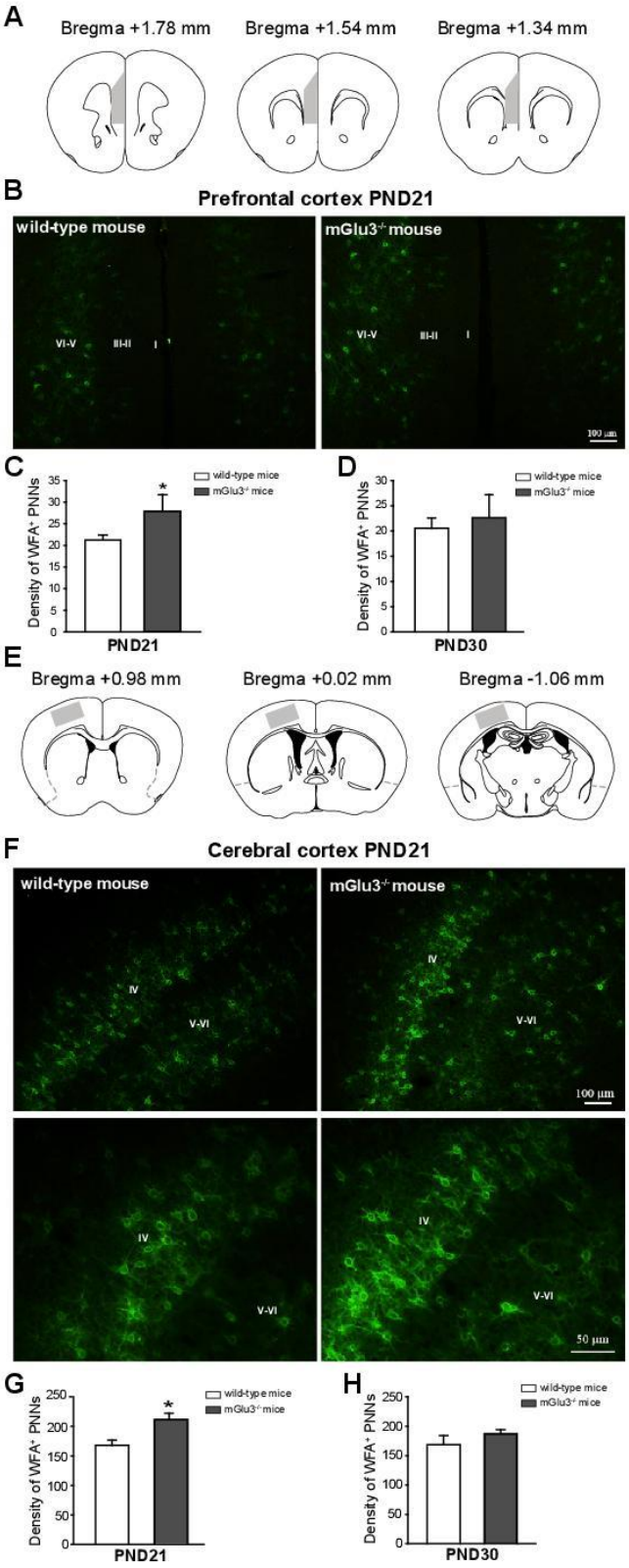

Figure 4. Increased density of WFA^+ PNNs in the cerebral cortex of $mGlu3^{-/-}$ mice at PND21. *A*) Fluorescent WFA staining of PNNs was performed in the medial portion of 3 sections containing the PFC (+1.78, +1.54, and +1.34 mm from bregma). Cell counting was performed at original magnification ×10 uniaterally in the portions highlighted in gray. *B*) Representative images of wild-type and $mGlu3^{-/-}$ mice at PND21 in the section at +1.54 from bregma, where cortical layers are indicated. *C*, *D*) Average density values of WFA^+ PNNs in the PFC of the 3 sections at PND21 and PND30, respectively. Values are means ± SEM of 3 mice/group in both graphs. *Statistically significant (Student's *t* test) *vs.* the respective wild-type mice; $t_4 = -2.87$; $P = 0.045$. *E*) WFA^+ cells were also counted unilaterally in the highlighted portions of the cerebral cortex in 3 sections at +0.98, +0.02, and −1.06 mm from bregma. *F*) Representative images at +0.02 mm from bregma, where cortical layers are also indicated. *G*, *H*) Average density values of WFA^+ PNNs in the cerebral cortex at PND21 and PND30, respectively. Values are means ± SEM of 4 mice/group at PND21 and 3 mice/group at PND30. *Statistically significant (Student's *t* test) *vs.* the respective wild-type mice; $t_6 = -6.514$; $P < 0.001$.

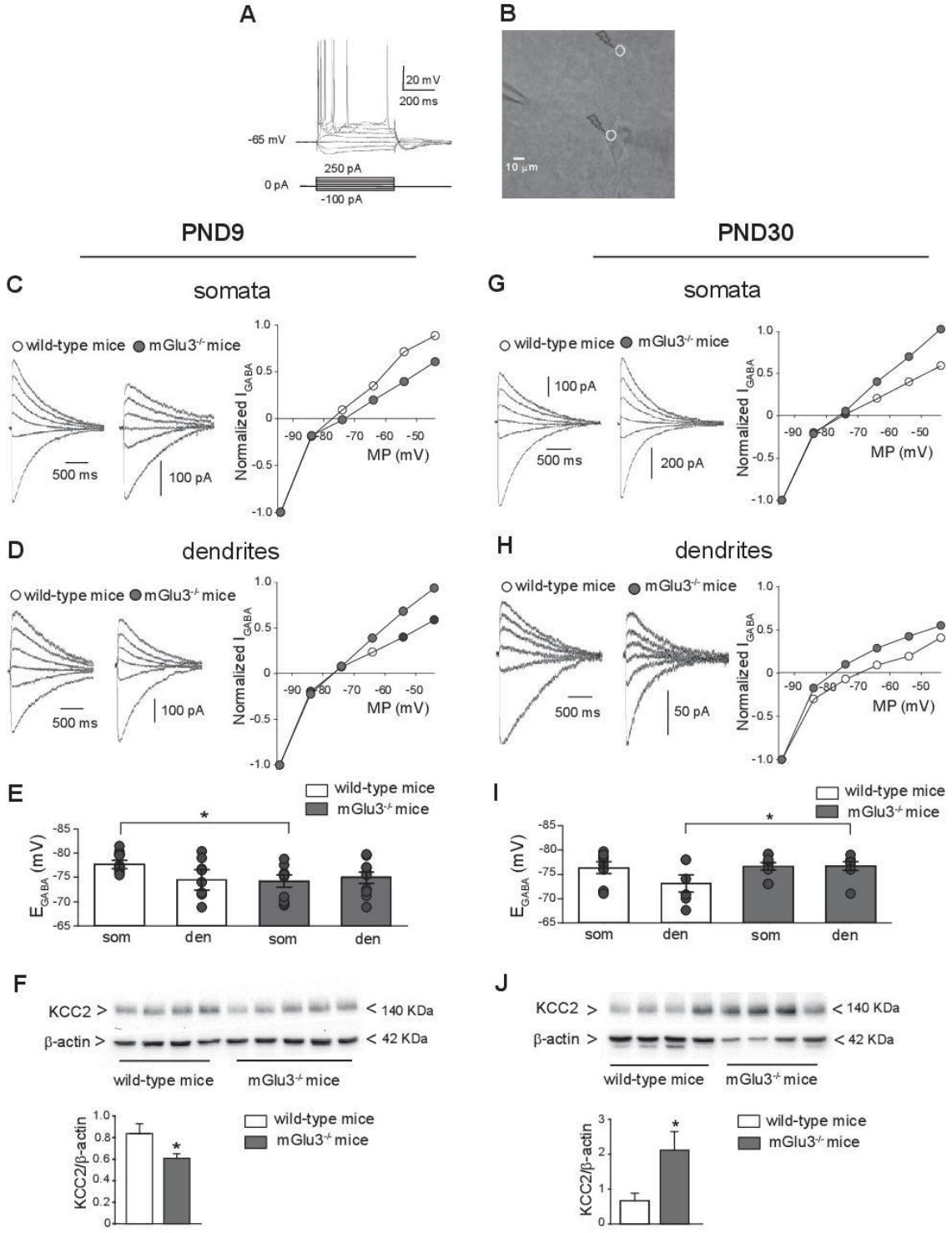

Figure 5. Effect of genetic deletion of mGlu3 receptors on $GABA_A$ receptor–mediated responses in pyramidal neurons of the PFC during postnatal development. *A*) Typical current-clamp recording of action potentials from an L5 pyramidal neuron in a slice obtained from the PFC. *B*) A microscopic image illustrating a RuBi GABA uncaging experiment, where circles represent the somatic (som) and dendritic (den) areas where laser-induced photostimulation was applied. *C, D, G, H*) Typical whole-cell I_{GABA} currents elicited by photolysis of RuBi GABA at different holding potentials and normalized *I-V* curves in som and den (about 50 µm from soma) from wild-type and mGlu3$^{-/-}$ mice at PND9 (*C, D*) and PND30 (*G, H*). *E, I*) Som and den E_{GABA} values in

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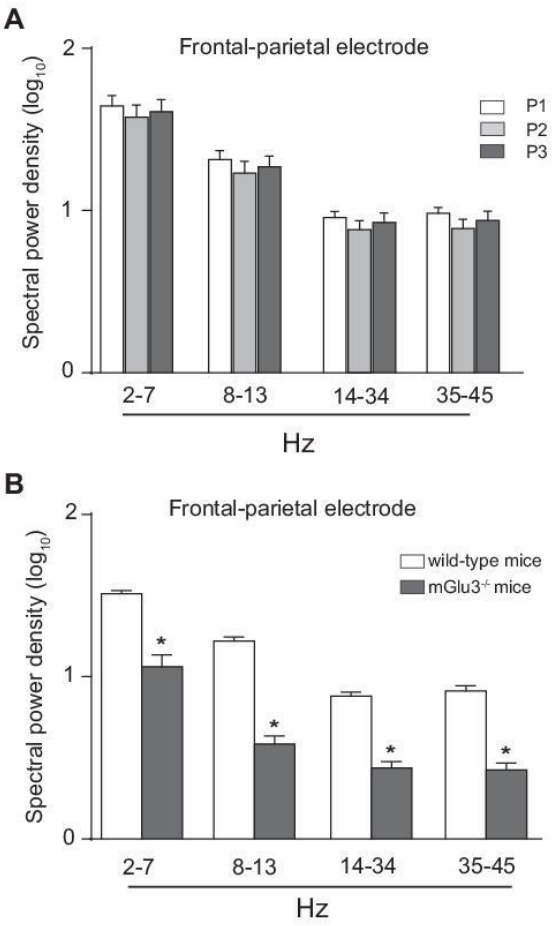

Figure 6. Effect of genetic deletion of mGlu3 receptors on EEG oscillatory activity in adult mice. *A*) Frontal-parietal EEG power density values in wild-type CD1 mice recorded in 3 time periods lasting 1 h (P1 = 8:00–9:00 PM; P2 = 9:00–10:00 PM; and P3 = 11:00–12:00 PM) in the light-off condition. All artifact-free 2-s EEG epochs were considered for the computation of those values. The frequency bands of interest were 2–7 Hz (δ-θ), 8–13 Hz (α), 14–34 Hz (β), and 35–45 Hz (γ). Values are means ± SEM of 7 mice. EEG power densities did not differ in the 3 time periods. *B*) EEG power density recorded in adult wild-type ($n = 7$) and mGlu3$^{-/-}$ ($n = 8$) mice during the P3 period. The data refer to ongoing EEG oscillations recorded during passive behavioral state in the quiet wakefulness. Values are means ± SEM. *Statistically significant *vs.* the respective values obtained in wild-type mice (1-way ANOVA + Duncan *post hoc* test). Genotype × frequency band interaction: $F = 10.6$; $P < 0.01$. *P* values (Duncan test): 2–7 Hz: $P = 0.025$; 8–13 Hz: $P = 0.001$; 14–34 Hz: $P = 0.004$; 35–45 Hz: $P = 0.001$.

GABAergic transmission in the cerebral cortex: *1*) the postnatal maturation of GABAergic interneurons, which acquire specific peptide-protein markers; *2*) the correct matching between pyramidal neurons and GABAergic interneurons (69); *3*) the GABA shift (*i.e.*, the developmental changes in neuronal chloride homeostasis driven by KCC2) (21); and *4*) the formation of PNNs, which restrain the plasticity of PV$^+$ interneurons and other neuronal types (70). Our data support the involvement of mGlu3 receptors in at least 3 of these mechanisms.

mGlu3$^{-/-}$ mice showed large reductions in the expression of PV and the GluN1 subunit of NMDA receptors, which lasted until the adult life (PND75). PV is a calcium-binding protein expressed by basket and chandelier cells, which innervate the soma and initial axonal segment of pyramidal neurons (71). NMDA receptors are highly expressed and constitutively active in PV$^+$ cells and other interneurons because the relatively low MP relieves the Mg^{2+} blockade of the ion channel (72). mGlu3$^{-/-}$ mice also showed a reduction in the transcripts encoding for VIP, Sst, and CB1 receptors at both PND1 and PND9. VIP is a specific marker of a subpopulation of interneurons that exclusively innervate other interneurons (73). Sst is a marker of Martinotti's cells that innervate the apical portion of pyramidal cell dendrites (74, 75). CB1 receptors are found in a subpopulation of PV$^-$ and cholecystokinine (CCK)$^+$ basket cells, which exhibit moderate accommodating firing patterns (76). Thus, it appears that the absence of mGlu3 receptors impairs the development of multiple populations of cortical GABAergic neurons. The reduction in Arx mRNA levels found in mGlu3$^{-/-}$ mice at PND1 is consistent with this hypothesis because Arx is essential for the development of cortical interneurons (77). We have also found that the transcript encoding for Reelin was up-regulated in the PFC of mGlu3$^{-/-}$ mice at PND1 and PND30. Reelin, a glycoprotein secreted from Cajal-Retzius cells in the embryonic life and from a subpopulation of GABAergic interneurons in the postnatal life, regulates neuronal migration in the developing cerebral cortex (78). Abnormalities in Reelin signaling are associated with psychiatry disorders (79), and a reduced expression of Reelin resulting from gene hypermethylation has been found in the brain of individuals affected by schizophrenia (80). Our data suggest that endogenous activation of mGlu3 receptors restrains Reelin expression in specific time windows during postnatal development, raising the attractive possibility that mGlu3 receptors are also involved in the regulation of neuronal migration.

The biochemical scenario of mGlu3$^{-/-}$ mice is similar to that observed in cortical tissue of individuals affected by schizophrenia, in which expression of PV, NMDA receptors, CCK, CB1 receptors, and other markers of GABAergic interneurons is consistently reduced without apparent changes in neuronal number (81–84). A reduced expression of NMDA receptors might be a primary event that drives other changes observed in mGlu3$^{-/-}$ mice.

wild-type and mGlu3$^{-/-}$ mice at PND9 (*E*) and PND30 (*I*). Values are means ± SEM of 6–9 determinations/group (obtained from 3 wild-type and 3 mGlu3$^{-/-}$ mice). Statistical analysis was performed by Student's *t* test by comparing E_{GABA} values recorded in the somata and in the dendrites separately. *Statistically significant *vs.* wild-type mice. PND9: $t_{19} = 2.295$; $P = 0.033$; PND30: $t_{11} = 2.946$; $P = 0.032$. *F, J*) Western blot analysis of KCC2 protein levels in the PFC of wild-type and mGlu3$^{-/-}$ mice at PND9 (*F*) and PND30 (*J*). Values are means ± SEM of 4–5 mice/group. Statistically significant *vs.* wild-type mice (Student's *t* test); $t_7 = 2.85$ (*F*); $P = 0.0245$; $t_6 = -2.569$; $P = 0.0424$ (*J*).

Accordingly, targeted deletion of the GluN1 subunit of NMDA receptors in cortical interneurons causes a reduced expression of GAD67 and PV, disinhibition of pyramidal neurons, and reduced synchrony in their activity and generation of EEG signals (85).

Another interesting finding was that KCC2 levels were reduced in the PFC of mGlu3$^{-/-}$ mice at PND9, and, consequently, the E_{GABA} measured in the soma of pyramidal neurons was more depolarized in these mice. $GABA_A$ receptors form Cl^--permeable ion channels that can either drive Cl^- influx or efflux depending on intracellular Cl^- concentrations. In the prenatal life, activation of $GABA_A$ receptors gives raise to excitatory postsynaptic potentials owing to the high intracellular levels of Cl^- in neurons. Expression of KCC2 in postnatal neurons lowers intracellular Cl^- concentrations, and activation of $GABA_A$ receptors leads to Cl^- influx, thereby mediating the inhibitory action of GABA in the mature brain. The more depolarized E_{GABA} found in the soma of pyramidal neurons of mGlu3$^{-/-}$ mice at PND9 suggests that, at this age, a dysfunction of PV$^+$ or CKK$^+$ interneurons, or both, may cause a down-regulation of KCC2 in their postsynaptic innervation territory in pyramidal neurons. Interestingly, KCC2 expression is reduced in the dorsolateral PFC of individuals affected by schizophrenia (86), and 1 rare loss-of-function variant of KCC2 (R952H) has been associated with schizophrenia (87). In addition, the developmental switch in $GABA_A$ receptor function is disrupted in 22q11.2 deletion syndrome, which is associated with high incidence of schizophrenia (88).

We were surprised to find that opposite changes (*i.e.*, a less depolarized E_{GABA}) were observed in dendrites of pyramidal neurons of mGlu3$^{-/-}$ mice at PND30, when an increase in PFC of KCC2 protein levels was also observed. Dendrites of pyramidal neurons are innervated by slow-spiking GABAergic interneurons, including Sst$^+$ interneurons. A less depolarized E_{GABA} in pyramidal neurons might further disrupt the balance between excitation and inhibition in the PFC of mGlu3$^{-/-}$ mice.

Postnatal maturation of PV$^+$ neurons is associated with the progressive build-up of PNNs, which are formed by a linear nonsulfated hyaluronic acid polymer joined to CSPGs through a series of linking proteins (22). PNNs enwrap about 15% of forebrain neurons, the majority of which are PV$^+$ (23, 24). In a first set of experiments, we stained PNN with Cat-315 antibodies, which binds to the human natural killer-1 (HNK-1) glycan epitope on aggrecan (23, 89, 90). The number of Cat-315$^+$ PNNs was higher in 3 sections of the cerebral cortex of mGlu3$^{-/-}$ mice at PND21, when PNNs begin to be fully expressed in the mouse cortex (25), but not at PND30. We failed to detect cells stained by Cat-315 antibodies in the PFC. For these reasons, we extended the analysis to PNNs stained with WFA. The lectin, WFA, binds to the chondroitin sulfate chains of aggrecan and is widely used to detect PNNs (91, 92). Cat-315 and WFA label distict subpopulations of PNNs (93). We could detect a small number of WFA$^+$ PNNs in the PFC, which was greater in mGlu3$^{-/-}$ mice at PND21. The density of WFA$^+$ PNNs was also greater in the 3 sections of the cerebral cortex used for the detection of Cat-315$^+$ PNNs in mGlu3$^{-/-}$ mice at PND21. Thus, the lack of mGlu3 receptors increased the number of 2 subsets of PNNs respectively labeled by Cat-315 and WFA at PND21, but not at PNF30. PNN deposition ensures structural stability of neuronal circuits encoding for long-term memory (26), and a reduction of PNNs have been found to improve cognitive flexibility (94, 95). Thus, an optimal level of PNNs within the appropriate time windows may be essential for the correct balance between excitation and inhibition underlying cognitive function. Our findings suggest that genetic dysfunction of mGlu3 receptors may enhance PNN formation and restrain the plasticity of PV$^+$ interneurons in advance with respect to the appropriate developmental time.

How might mGlu3 receptors affect the postnatal development of cortical GABAergic transmission? Traditionally, the mGlu3 receptors have been considered as presynaptic receptors that negatively modulate neurotransmitter release (3). However, electron microscopy data have shown that mGlu3 receptors are concentrated in postsynaptic elements in the PFC (96) and are involved in mechanisms of activity-dependent synaptic plasticity underlying PFC-dependent cognitive functions (97). Polymorphic variants of GRM3 in humans are associated with low performance in PFC-dependent cognitive tasks (98, 99). mGlu3 receptor activation enhanced mGlu5 receptor–mediated Ca^{2+} mobilization in PFC pyramidal neurons, and mGlu3 receptor-dependent LTD in the PFC required the endogenous activation of mGlu5 receptors (15). It is possible that the lack of mGlu3 receptors causes a primary dysfunction in pyramidal neurons, which alters the excitatory control of GABAergic interneurons. Alternatively, an interaction between mGlu3 and mGlu5 receptors may also take place in interneurons, regulating mechanisms that are essential for their development. mGlu3 receptors present in glial cells might also contribute to the development of cortical interneurons. In astrocytes, mGlu3 receptor activation stimulates the production of glial cell line–derived neurotrophic factor (100, 101), which is involved in the early development of GABAergic interneurons (102, 103). Which of the above mechanisms contributes to the regulation of Cat-315$^+$ and WFA$^+$ PNN formation in the cerebral cortex is unknown. It is possible that endogenous activation of mGlu3 receptors in neurons or astrocytes modulates the expression of enzymes involved in the formation or degradation, or both, of PNNs. This attractive hypothesis warrants further investigation.

We also performed EEG experiments in mGlu3$^{-/-}$ and wild-type mice to examine whether genetic deletion of mGlu3 receptors may have a permanent effect on neuronal synchronization and desynchronization mechanisms regulating brain arousal in quiet wakefulness. This behavioral mode is reliably detectable in mice when compared with phases of active exploration of the cage, instinctual movements, or sleep. Results of these experiments showed that the power of frontal-parietal EEG oscillations in a wide frequency range from δ to γ (2–45 Hz) was markedly reduced in mGlu3$^{-/-}$ mice as compared with their wild-type counterparts. Because of the novelty of our EEG data obtained in mGlu3$^{-/-}$ mice, and because of the lack of intracerebral recording of local field potentials from the thalamus and other relevant subcortical

structures of the ascending reticular activating systems, the interpretation of these findings can only be speculative and relies on the following observations reported in previous investigations:. cortical EEG oscillations are sustained by a corticothalamic-cortical loop, in which GABAergic neurons of the RTN regulate the firing rate of thalamocortical neurons by influencing the inactivation kinetics of T-type voltage-sensitive Ca^{2+} channels with effects on cortical EEG rhythms from δ to σ frequencies (<14 Hz) in the sleep-wake cycle (104–107). In this loop, mGlu3 receptors are present in RTN neurons (16), and were found in excitatory and inhibitory presynaptic terminals of ventrobasal thalamic nuclei (17); and network activity in the cerebral cortex is shaped by cortical interneurons and their reciprocal connections with pyramidal neurons. Specifically, pyramidal neurons and PV^+ interneurons (mainly basket cells) generate γ oscillation through the pyramidal interneuron network γ model in which asynchronously firing pyramidal neurons excite PV^+ interneurons, which, in turn, silence pyramidal neurons. Synchronous decay of inhibition allows the coordinated firing of a large group of pyramidal neurons, which, in turn, excite PV^+ interneurons giving rise to oscillatory activity in the γ frequency band (82). Because inhibitory postsynaptic currents mediated by $GABA_A$ receptors at synapses between CCK-containing basket cells and pyramidal neurons have a slow decay, it is believed that CCK^+ interneurons are mainly involved in the generation of θ oscillations (108, 109). Thus, the lack of mGlu3 receptors at different synapses at both cortical and thalamic levels may explain the reduced frontal-parietal EEG oscillations found in $mGlu3^{-/-}$ mice. A life-long dysfunction of NMDA receptors (suggested by the reduced expression of the GluN1 subunit at PND75), which mediate synaptic excitation at multiple poopulations of cortical interneurons, might also contribute to EEG changes found in $mGlu3^{-/-}$ mice.

Our findings give impetus to future studies testing the hypothesis that the reported EEG alterations might be related to the psychotic-like phenotype exhibited by $mGlu3^{-/-}$ mice (10, 11, 110). Interestingly, individuals affected by schizophrenia consistently show EEG abnormalities in γ and θ oscillations both under basal conditions and during sensory processing or execution of cognitive tasks (36, 111–114), as a possible result of a dysfunction of cortical GABAergic interneurons.

In summary, the present findings demonstrate that mGlu3 receptors are required for a proper development of GABAergic neurotransmission in the PFC and suggest that a dysfunction of these receptors alters the developmental trajectory of cortical GABAergic interneurons. They also suggest an association between such a dysfunction and long-lasting changes in thalamic and cortical networks underpinning quiet wakefulness and generation of EEG oscillations. It will be interesting to test the hypothesis that the genetic variants of GRM3 associated with psychiatric disorders alter the developmental trajectory of inhibitory neurotransmission in the PFC by causing changes in the expression or function of mGlu3 receptors. If so, an early treatment with drugs that selectively amplify mGlu3 receptor function in the appropriate temporal windows may be beneficial in individuals with hypomorphic GRM3 mutations.

This early treatment might represent a new strategy to correct the developmental abnormalities of GABAergic transmission associated with schizophrenia, taking into account that drugs that do not display cellular specificity in enhancing GABAergic transmission (*e.g.*, benzodiazepines or other $GABA_A$ receptor PAMs) are not effective in the treatment of psychotic disorders. A dysfunction of GABAergic interneurons of the PFC has been implicated in the pathophysiology of depression and stress-related disorders (115, 116), and mGlu2/3 receptor antagonists display antidepressant-like activity in animal models and are under clinical development for the treatment of major depression (117, 118). Thus, our findings may encourage the preclinical development of mGlu3 receptor negative allosteric modulators as potential antidepressant agents. FJ

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AUTHOR CONTRIBUTIONS

T. Imbriglio, R. Verhaeghe, K. Martinello, M. T. Pascarelli, G. Chece, D. Bucci, S. Notartomaso, M. Quattromani, G. Mascio, and F. Scalabrì performed research; A. Simeone, S. Maccari, and T. Wieloch contributed new reagents or analytic tools; C. Del Percio, S. Fucile, C. Babiloni, G. Battaglia, and C. Limatola analyzed data; C. Babiloni, F. Nicoletti, and M. Cannella wrote the paper; and F. Nicoletti and M. Cannella designed the research.

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CHAPTER 3

INTERPLAY BETWEEN MGLU3 RECEPTORS AND MATERNAL RESTRAINT STRESS ON BRAIN DEVELOPMENT

Most diseases are complex and stem from an interaction between genes and environment. Environment and experiences vary from one individual to another. Together with genes, they shape individual behavior. The last part of my thesis was dedicated to study the interplay between genes and environmental factors such as maternal restraint stress (MRS).
Early life stress impairs critically the glutamatergic synapse, but also GABAergic system as shown by growing evidences. Indeed, early life stress decreases GABA receptors expression subunit (*Nejatbakhsh et al., 2019; Laloux et al., 2012*), GABAergic markers such as reelin, GAD67 and GAD65 (*Matrisciano et al., 2012, 2013*; *Stone et la., 2001*), GAD^+ neurons (*Grigoryan and Segal, 2013*) and delays GABA cell migration (*Stevens et al., 2013*). Other studies found a reduction of PV^+ interneurons density in hippocampus in heterozygous GAD67-GFP (Knock-In; $GAD67^{+/GFP}$) mice subjected to prenatal stress but not in naïve mice and wilt-type mice suggesting that interplay between genes and environment induce this phenotype (*Wang et al., 2018*; *Uchida et al., 2014*).

Since we previously reported that the deletion of mGlu3 receptor in mice lead to developmental abnormalities in cortical GABAergic system, we wanted to further investigate how the deletion of the mGlu3 receptors coupled with MRS could impact the GABAergic system as well stress related genes and epigenetic markers during development in hippocampus, a central brain region in stress response.

In order to study the interplay between nature and nurture (eg. genes and environment) we submitted $mGlu3^{-/-}$and wild-type mice to a prenatal restraint stress from embryologic day 15 to embryologic day 17.5. Then, we analyzed several GABAergic interneurons markers, stress markers and epigenetic marks in hippocampus by real-time PCR in early postnatal life.

Interplay between mGlu3 receptors and maternal restraint stress on the development of neuroplasticity genes expression in the hippocampus

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ABSTRACT

The developmental trajectory of cortical GABAergic transmission in postnatal life is influenced by mGlu3 receptors. Because genes and environment shape the brain development, we studied the interplay between these risk factors on the expression of GABAergic interneuron-related genes in the hippocampus, a target region of early life stress. Hence, in the present study, we used mGlu3$^{-/-}$ mice and wild type mice that underwent maternal restraint stress (MRS) from embryonic days 14.5 to 17.5, a critical period for GABAergic interneurons maturation and migration. We showed that genetic deletion of mGlu3 receptors decreased the transcripts encoding parvalbumin, calretinin, neuropeptide Y, and GAD67 at PND21. MRS induced large reductions in somatostatin, cholecystokinin, GABA transporter 1 and glutamic acid decarboxylase 67 gene expression at PND30. We also showed that MRS consistently increased transcripts encoding reelin and GluN1 at PND9 and PND21. Moreover, changes of stress markers as well as epigenetic marks in mGlu3$^{-/-}$ mice and MRS mice have been observed, confirming the hippocampus as a main target of maternal stress, which is known to induce epigenetic alterations in the offspring. Interestingly, we showed that MRS in mGlu3$^{-/-}$ mice induced compensatory and protective effects on GABAergic interneurons related genes expression. Together these results strengthen not only the important role of mGlu3 receptors but also the influence of the interplay between mGlu3 receptors and MRS on the development of GABAergic interneurons in the hippocampus.

Keywords: mGlu3 knockout mice, hippocampus, GABAergic interneurons, epigenetic markers, glucocorticoids MR/GR, BDNF

INTRODUCTION

Genes and environment, acting in concert, shape the developmental trajectory of the CNS (Plomin and Daniels, 1987; Stiles, 2011; Weaver 2014), and exposure to adverse environmental conditions during critical developmental time windows may cause long-lasting effects on brain function and behavior (Maccari et al., 2017). A large body of evidence suggests that perinatal stress may have long-term programming effects on the brain, depending on the timing and extent of exposure to stressful conditions (Bock et al., 2015; Maccari et al., 2014; Brunton and Russell, 2008; Matrisciano et al., 2012). In line with this hypothesis, the PRS mouse model has been shown to induce behavioral and molecular neuroepigenetic alterations (Dong et al., 2016), which could be targeted by conventional antipsychotic agents or by drugs that activate type 2/3 metabotropic glutamate (mGlu2/3 receptors (Matrisciano et al., 2012; Matrisciano et al., 2013).

mGlu receptors regulate GABAergic transmission in different brain regions (reviewed by Nicletti et al., 2011), and recent findings suggest that they can also regulate the development of GABAergic interneurons in the CNS. Although GABAergic interneurons represent only 10-15% of hippocampal neurons, they are critically involved in the regulation of network oscillations because they tune the synchronization of pyramidal cells (the principal excitatory neurons) and therefore shape information processing in the hippocampus. Regulation of pyramidal cell synchronization is a key process in the coordinated activity of neuronal networks underlying higher brain function. Specific subpopulations of GABAergic interneurons, such as parvalbumin-positive (PV^+) interneurons are critical for this function (Uhlhaas et al., 2009; Pelkey et al., 2017). Disruption of mGlu5 receptors from PV^+ interneurons decreased of the numbers of interneurons and inhibitory currents recorded from pyramidal neurons in the cerebral cortex. These changes were associated with increased compulsive-like behaviors, abnormal sensorimotor gating and altered responsiveness to psychostimulants (Barnes et al., 2015). mGlu3 receptors are functionally linked to mGlu5 receptors, and their activation boost mGlu5 receptor signaling in the cerebral cortex, hippocampus, and other brain regions (Di Menna et al, 2018). Interestingly, genetic deletion of mGlu3 receptors in mice lead to behavioral alterations such as hyperactivity in the open field, light/dark transition, and impaired working memory and contextual memory (Fujioka et al., 2014; Lainiola et al., 2014). We recently found that the deletion of mGlu3 receptors in mice have a strong impact on the development of cortical GABAergic transmission in the prefrontal cortex (Imbriglio, Verhaeghe et al., 2019). $mGlu3^{-/-}$ mice showed robust changes in

the expression of interneuron-related genes in the prefrontal cortex, including large reductions in the expression of parvalbumin and the GluN1 subunit of NMDA receptors. The number of cortical cells enwrapped by perineuronal nets was increased in mGlu3$^{-/-}$ mice and adult mGlu3$^{-/-}$ mice showed lower power in electroencephalographic rhythms (Imbriglio, Verhaeghe et al., 2019).

Stress response is mediated by glucocorticoids and glutamate and stress are tightly connected (Popoli et al., 2011). For example, hippocampal mGlu2 receptors regulate resilience to stress through mechanisms that involve the epigenetic regulation of mineralcorticoid receptors (MR) (Nasca et al., 2014; 2017). However, little is known on how genes and environment interact in the development of GABAergic system. This gave us the impetus to investigate how deletion of the mGlu3 receptors combines with MRS in regulating the expression of GABAergic-related neurons in the hippocampus, the key brain region in the response to stress (McEwen, 1999; Maccari et al., 2017). We also analyzed genes transcript encoding for glucocorticoids receptors (Nr3c1), mineralocorticoids receptors (Nr3c2) as well brain derived neurotrophic factor (BDNF) that play a key role in plasticity induced by stress. To this purpose, we submitted mGlu3$^{-/-}$and wild-type mice to a MRS from embryologic day 14.5 to embryologic day 17.5, a critical time window for formation and migration of GABAergic interneurons in the fetal brain. Then, we analyzed several GABAergic interneurons markers, stress markers and epigenetic marks in hippocampus by real-time PCR across the postnatal development.

MATERIAL AND METHOD

Animals

Adult male and female CD1 mice were housed under controlled conditions (T = 22°C; humidity = 40%) on a 12 h light-dark cycle with food and water *ad libitum*. Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health to minimize the number of animals and animal suffering. The experimental protocol was approved by the Ethical Committee of Neuromed Institute (Pozzilli, Italy) and by the Italian Ministry of Health. mGlu3 receptor knockout mice (mGlu3$^{-/-}$ mice) with a CD1 genetic background were kindly provided by Eli Lilly & Company, Indianapolis, IN. One male and two females of mGlu3$^{-/-}$, and wild-type CD1 mice were housed for homozygous mating. We did not use littermates generated by heterozygous mating to minimize the number of mice

(experiments were performed exclusively on homozygous mice). Pups at postnatal days (PNDs) 9, 21, and 30 were used for biochemical analysis of selected genes in the hippocampal area.

Maternal restraint stress (MRS)

The stress procedures were used as described (a widely used restraint-and-light stress procedure (Montano et al. 1993; Fujioka et al. 2006; Uchida et al. 2011), on pregnant mice. The stress was performed three times a day for 45 min per session (08:30-09:15, 12:30-13:15, 16:30-17:15) from E14.5 to17.5 with a transparent plastic tube with a diameter of 3 cm. The total number of stresses per a mouse was nine. After the last stress treatment, the pregnant mice were not disturbed until the due date. In the non stressed and stressed groups, progeny from at least three pregnant mice were used for the experiments.

Measurements of mRNA levels of interneuron-related genes

Mice were killed by decapitation at PND 9, 21 or 30. The brains were removed and the hippocampus immediately dissected and frozen on liquid nitrogen. Total RNA was extracted using the trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The RNA was further treated with DNAse (Qiagen, Hilden, Germany), and single strand cDNA was synthesized from 1.5 μg of total RNA using Superscript III (Invitrogen) and random hexamers. Real-time PCR was performed on 15 ng of cDNA by using specific primers and Power SYBR Green Master Mix (Biorad, Hercules, CA) on an Applied Biosystems Step-One instrument.

Thermal cycler conditions were as follows: 10 min at 95°C, 40 cycles of denaturation (15 sec at 95°C), and combined annealing/extension (1 min at 58-60°C). Primers sequences of all genes are reported in Supplementary Table 1. mRNA copy number for each gene was calculated from serially diluted standard curves simultaneously amplified with the samples and normalized with respect to the transferrin receptor (TFRC) mRNA copy number. Each sample was analyzed in duplicate together with two negative controls.

RESULTS

We examined whether the lack of mGlu3, MRS or the combination of mGlu3 deletion and MRS could affect the expression of genes encoding for 1) biochemical markers of

GABAergic interneurons, 2) stress response-related genes and 3) epigenetic marks in the hippocampus in the first month of postnatal life (at PND9, PND21 and PND30).

At post-natal day 9 (PND9) (**Fig. 1A**), MRS in wild type and mGlu3$^{-/-}$ mice induced an increase in the transcripts of the genes encoding neuropeptide Y (NPY) and cannabinoid receptor type1 (Cnr1). MRS in wild-type mice selectively increased the transcripts of reelin (Reln) and GluN1 (Grin1). Furthermore, Reln mRNA levels were higher in unstressed mGlu3$^{-/-}$ mice in unstressed wild type mice. Interestingly, parvalbumin (Pvalb) transcript levels were selectively increased in mGlu3$^{-/-}$ MRS mice.

At PND21, we observed substantial changes in the expression of GABAergic neuron-related genes in our experimental groups (**Fig. 1B**). Transcript analysis revealed a down-regulation of Pvalb, calretinin (Calb2), NPY in mGlu3$^{-/-}$ non-MRS mice compared to wild type non-MRS mice. In addition, we found an increase in somatostatin (Sst), Reln, cholecystokinin (CCK), GABA transporter 1 (Slc6a1), glutamate decarboxylase 65 (GAD65) mRNA levels in mGlu3$^{-/-}$ non-MRS mice with respect to wild type non-MRS mice. Mice carrying both mGlu3 deletion and submitted to MRS displayed an increase in transcripts encoding Pvalb, Calb2, CCK and NPY with respect to mGlu3$^{-/-}$non-MRS mice. In contrast, transcript levels of Reln, Slc6a1, GAD65, vasoactive intestinal peptide (VIP) and Sst were lower in mGlu3$^{-/-}$ MRS mice than in mGlu3$^{-/-}$ non-MRS mice. Wild type mice submitted to MRS showed a decrease of Calb2, Slc6c1, VIP mRNA levels compared to wild type non MRS mice. The transcripts encoding Sst, Grin1, Reln, CCK, Slc6a1 were instead higher in mGlu3$^{-/-}$ MRS mice than in wild type MRS mice.

At PND30, (**Fig. 1C**) Sst, CCK and Slc6a1 transcripts were reduced by MRS in both wild type and mGlu3$^{-/-}$ mice. mGlu3$^{-/-}$non MRS mice displayed increases in Cnr1 and GAD65 mRNA levels compared to wild type non MRS mice. Moreover, GAD65 mRNA levels were lower in mGlu3$^{-/-}$ MRS mice than in mGlu3$^{-/-}$ non MRS mice. MRS reduced GAD67 transcripts selectively in wild type mice.

We analyzed genes transcript encoding for glucocorticoids receptors (Nr3c1), mineralocorticoids receptors (Nr3c2) and BDNF, which are involved in mechanisms of neuroadaptation induced by maternal stress. At PND9 (**Fig. 2A**), mGlu3$^{-/-}$ non-MRS mice showed an increase in Nr3c1, Nr3c2, and BDNF with respect to wild-type non-MRS mice. In addition, Nr3c2 mRNA levels were also higher in wild type MRS mice compared to wild type non-MRS mice. The analysis of stress-related genes at PND21 (**Fig. 2B**) revealed lower levels

of Nr3c1 and BDNF in mGlu3$^{-/-}$ non-MRS mice compared to wild type non-MRS mice. MRS also reduced BDNF mRNA selectively in wild-type mice. Furthermore, we found an increase in Nr3c2 in mGlu3$^{-/-}$ MRS mice with respect to wild type MRS mice. Among transcripts encoding stress markers at PND30 (**Fig. 2C**), Nr3c1 mRNA levels were reduced by maternal stress in both wild type mice and mGlu3$^{-/-}$ mice. Furthermore, mGlu3$^{-/-}$ non MRS mice displayed lower Nr3c1 transcript levels than wild type non MRS mice. BDNF gene expression was also decreased in mGlu3$^{-/-}$ MRS mice compared to mGlu3$^{-/-}$ non-MRS mice. We further analyzed several epigenetic markers. At PND9, we observed an increase in methyl CpG binding protein 2 (Mecp2), DNA (cytosine-5)-methyltransferase 1 (DNMT1) and histone deacetylase 3 (HDAC3) transcripts in mGlu3$^{-/-}$ non MRS mice and wild type MRS mice compared to wild type non MRS mice (**Fig. 2C**). At PND21, MRS caused substantial changes in the transcripts of epigenetic markers (**Fig. 2D**) as demonstrated by the increase of Mecp2 and HDAC3 mRNA levels in mGlu3$^{-/-}$ MRS mice compared to mGlu3$^{-/-}$ non-MRS mice. Notably, HDAC3 mRNA levels were significantly higher in mGlu3$^{-/-}$ non-MRS mice than in wild type MRS mice Moreover, MRS reduced DNMT1 mRNA levels selectively in wild type mice. At PND30, MRS reduced DNMT1 and HDAC3 mRNA levels in both wild type non-MRS mice and mGlu3$^{-/-}$ non-MRS mice (**Fig. 2E**).

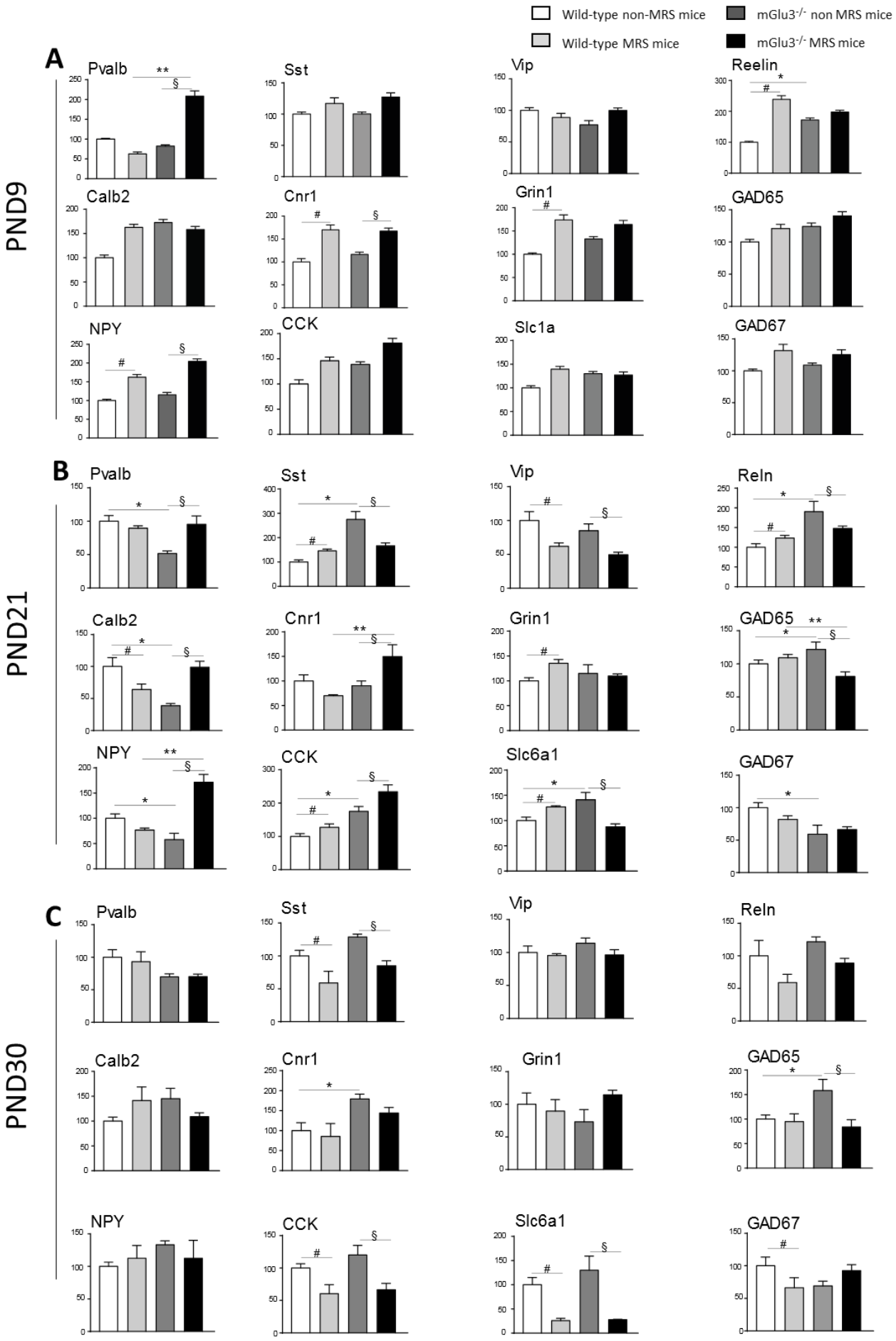

Figure 1 - Influence of genetic deletion mGlu3 receptors and maternal restraint stress on biochemical markers of GABAergic interneurons in the hippocampus at PND9 (**A)**, PND21 (**B)** and PND30 (**C)**. (**A**) Cnr1, MRS $F_{(3;30)}$ =11.23 wild-type p=0.013, mGlu3$^{-/-}$p=0.047; Npy, $F_{(3;\ 29)}$= 15.35; Nr3c1, $F_{(3;\ 19)}$= 9.2; Nr3c2, $F_{(3;\ 19)}$= 5.52; Pvalb, MRS mGlu3$^{-/-}$ $F_{(3;\ 32)}$ = 19.03 p<0.001; genotype x MRS $F_{(3;\ 32)}$ =30.90 p<0.001; Reln, genotype $F_{(3;28)}$= 8.94 p=0.006; MRS wild-type $F_{(3;\ 28)}$=18.6 p <0.001; Grin1, $F_{(3;31)}$= 8.97**;** (**B**) Calb2, $F_{(3;\ 23)}$ = 28.83; CCK, $F_{(3;\ 21)}$ = 49.60; Cnr1, $F_{(3;\ 23)}$ = 7.72; Gad_{67} $F_{(3;\ 23)}$ = 12.04; Gad_{65} $F_{(3;\ 23)}$ = 12.74; Grin1, $F_{(3;\ 22)}$ = 4.6; ; Npy, $F_{(3;\ 22)}$ = 50.95; ; Pvalb, $F_{(3;\ 23)}$= 15.05; Reln, $F_{(3;\ 22)}$= 29.04; Slc6a1, $F_{(3;\ 22)}$= 19.14; Sst, $F_{(3;\ 23)}$= 31.15; Vip, $F_{(3;\ 23)}$ = 19.97; (**C**) CCK, $F_{(3;\ 19)}$ = 19.19; Cnr1, $F_{(3;\ 15)}$ = 12.66; ; Gad_{67} $F_{(3;\ 18)}$ = 6.57; Gad_{65} $F_{(3;\ 18)}$= 6.64; Pvalb, $F_{(3;\ 19)}$= 10.64; Reln, $F_{(3;\ 18)}$= 8.63; Slc6a1, $F_{(3;\ 18)}$= 62.32; Sst, $F_{(3;\ 18)}$ = 17.87.

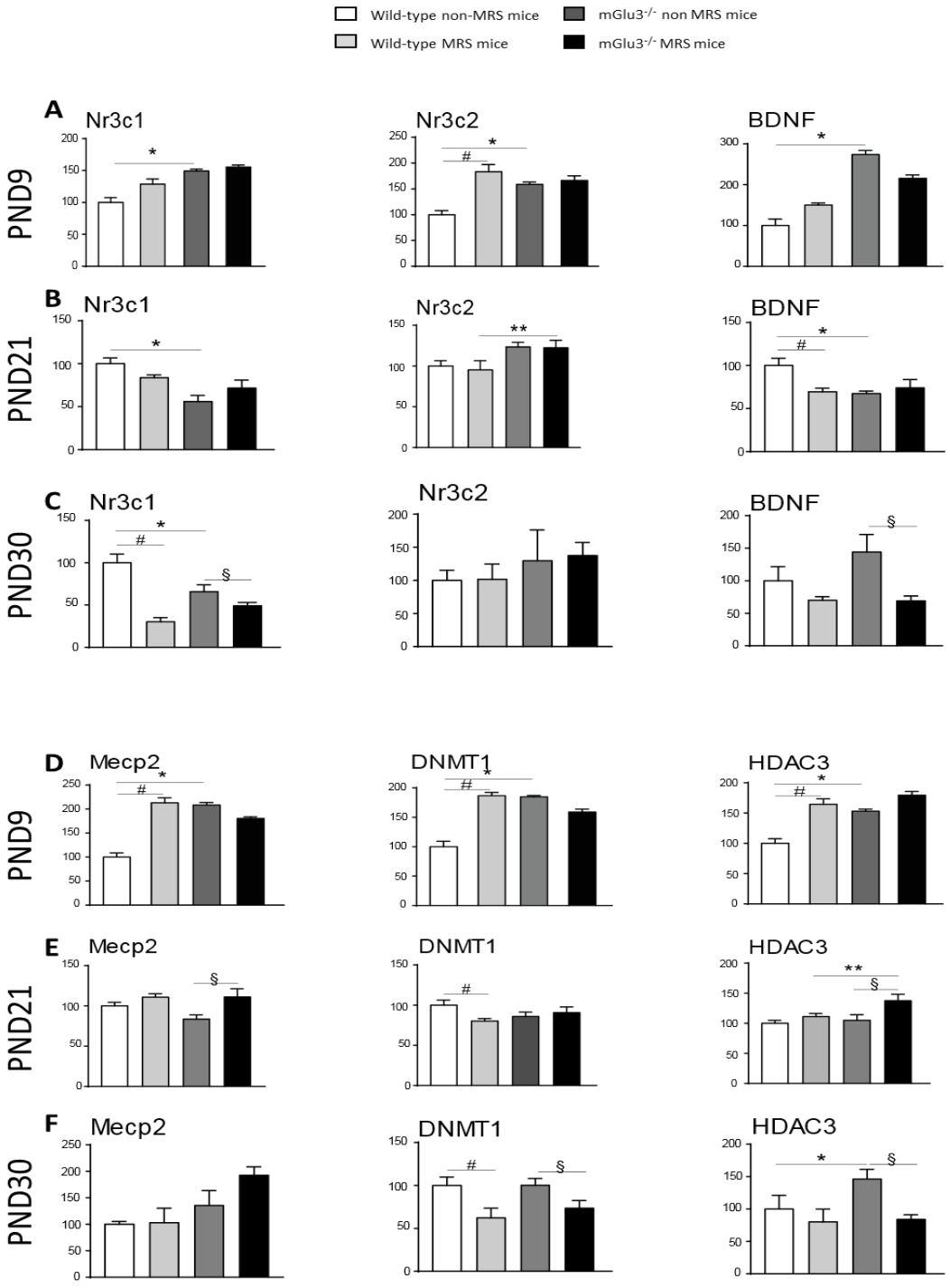

Figure 2- Influence of genetic deletion mGlu3 receptors and maternal restraint stress on biochemical markers of stress response in the hippocampus at PND9 (**A)**, PND21 (**B)** and PND30 (**C)**. (**A**) BDNF, $F_{(3;\ 19)}$ = 23.97; (**B**) BDNF, $F_{(3;\ 23)}$ = 8.18; Nr3c1, $F_{(3;\ 23)}$= 21.76; Nr3c2, $F_{(3;\ 23)}$ = 8.54; (**C**) BDNF, $F_{(3;\ 18)}$ = 11.37; Nr3c1, $F_{(3;\ 19)}$= 55.21.
Influence of genetic deletion mGlu3 receptors and maternal restraint stress on epigenetic marks in the hippocampus at PND9 (**D)**, PND21 (**E)** and PND30 (**F)**. (**A**) DNMT1, $F_{(3;19)}$=17.4; HDAC3, genotype $F_{(3;19)}$=5.6 p=0.029; MRS $F_{(3;19)}$= 9.83 p=0.005; Mecp2, $F_{(3;\ 19)}$= 20.62; (**B**) DNMT1, $F_{(3;\ 22)}$ = 5.64HDAC3, $F_{(3;\ 23)}$= 10.24; Mecp2, $F_{(3;\ 23)}$= 9.78; (**C**) DNMT1, $F_{(3;\ 19)}$ = 12.96; HDAC3, $F_{(3;\ 19)}$= 9.05; Mecp2, $F_{(3;\ 19)}$= 10.65.

DISCUSSION

Our results strengthen not only the important role of mGlu3 receptors but also the influence of the interplay between mGlu3 receptors and MRS on the development of GABAergic interneurons in the hippocampus. Indeed, we showed that $mGlu3^{-/-}$ mice showed reduced parvalbumin mRNA levelsat PND21 and a trend to a reduction at PND30. Parvalbumin is a calcium-binding protein expressed by basket and chandelier cells, which innervate the soma and initial axonal segment of pyramidal neurons (Hu et al., 2014). Parvalbumin is believed to be a convergence point of genetic and environmental factors and our data fully support this hypothesis (Jiang et al., 2013). Interestingly, our results showed that cumulative $mGlu3^{-/-}$ deletion and MRS increased parvalbumin transcripts at PND9 and PND21, showing that gene x environment induces different changes on parvalbumin transcripts with respect to MRS or mGlu3 receptor deletion alone. PV^{+} and somatostatin $(Sst)^{+}$ interneurons cover 70% of the total population of GABAergic interneurons (Booker and Vida, 2018). Sst^{+} interneurons innervate the apical portion of pyramidal cell dendrites (Chiu et al., 2013). Although, somatostatin transcripts differ depending on MRS and $mGlu3^{-/-}$ deletion we still found a significant decrease in $mGlu3^{-/-}$ MRS mice at PND21 and at PND30. Interestingly, we also observed a diminution of VIP mRNA induced by MRS at PND21. VIP is a specific marker of a subpopulation of interneurons that exclusively innervate other interneurons (Pi et al., 2013). Transcripts encoding for reelin were consistently increased in wild type MRS mice and $mGlu3^{-/-}$ mice compared to wild type non stressed mice. Reelin regulates processes of neuronal migration and abnormal expression of Reelin can lead to neurodevelopmental disorders (Folsom and Fatemi, 2012). Moreover, MRS induced important reduction of somatostatin, cholecystokinin, GABA transporter 1 and glutamic acid decarboxylase 67 gene expression at PND30.

MRS selectively induced in wild-type mice an increase in the transcript encoding the GluN1, subunit of NMDA receptors (Traynelis et al., 2010; Sengar et al., 2019). As activation of NMDA receptors regulates both glutamatergic (Paoletti et al., 2013) and GABAergic transmission (Xue et al., 2011), these results suggest that MRS could alter the excitatory/inhibitory balance leading potentially to abnormalities in neuronal network.

Interestingly we observed different transcripts profile for GAD65 and GAD67. GAD65 was upregulated in mGlu3$^{-/-}$ non-MRS mice compared to wild type non-MRS mice at PND21 and PND30, whereas GAD67 transcripts levels were lower in mGlu3$^{-/-}$ non-MRS mice and wild type stressed mice than wild type non-stressed mice at PND21 and PND30, respectively. GAD65 and GAD67 are two isoforms of glutamic acid decarboxylase (GAD), an enzyme involved in GABA synthesis. Although both isoforms are present in most GABAergic interneurons in the central nervous system, GAD65 and GAD67 are believed to fulfill distinct role as demonstrated by their different location in the cell and their different expression during brain development. GAD67 predominates early during development whereas GAD65 is expressed later (Pinal and Tobin 1998; Soghomonian and Martin, 1998). Importantly, one of the most consistent findings in postmortem brain studies of schizophrenics is the decreased expression of the 67-kDa isoform of glutamate decarboxylase (GAD67), the primary GABA-synthesizing enzyme, in a subpopulation of GABAergic neurons (Guidotti *et al*, 2000; Hashimoto *et al*, 2003; Volk *et al*, 2000; Heckers and Konradi, 2015). In addition to change in GAD65 and GAD67 gene expression, we showed that the transcript of Slc6a1 encoding the high affinity GABA transporter, GAT1, was decreased by MRS in mGlu3$^{-/-}$ mice at PND21 and in wild type and mGlu3$^{-/-}$ mice at PND30. Taken together, these data suggest that the combination of mGlu3 receptor deletion and MRS impairs GABA metabolism and synaptic clearance.

Given that the programming effect of MRS is mediated by glucocorticoids (Maccari et al., 1995; Maccari et al., 2014; Maccari et al., 2017), it is not surprising that the glutamatergic system and glucocorticoids are tightly associated (Popoli et al., 2011). Because maternal stress is known to alter the GABAergic system in the adult life (Murthy et al., 2019), we analyzed Nr3c1 and Nr3c2 encoding GRs and MRs, respectively. GRs and MRs in the hippocampus are essential for stress response because they mediate the negative feedback within the HPA axis (Sapolsky and McEwen, 1984; Sapolsky et al., 1984; McEwen, 2001). We found that MR was upregulated in wild type MRS mice and mGlu3$^{-/-}$ non-stressed mice at PND9 whereas MR transcripts levels were higher only in mGlu3$^{-/-}$ stressed mice at PND21.

On the other hand, GR transcripts were up-regulated in mGlu3$^{-/-}$ non-MRS mice whereas they were down-regulated in mGlu3$^{-/-}$ non-MRS mice at PND21 and PND30. Of note, MRS decreased GR mRNA at PND30. Reduction of GRs in the hippocampus following perinatal restraint stress has been found in several studies (Maccari et al., 1995; Van Waess et al., 2011) and this suggests that short maternal stress for 3 days is also sufficient to alter the development of glucocorticoid receptors. Furthermore, stress regulates BDNF (Egan et al., 2003; Rasmusson et al., 2002), which interacts with the GABAergic system (Vaz et al., 2011). Here we showed that the deletion of mGlu3 receptors increased BDNF transcripts levels in non-MRS mice at PND9, but decreased BDNF mRNA levels at PND21. Interestingly, MRS reduced BDNF selectively in mGlu3$^{-/-}$ mice at PND21 and decreased BDNF mRNA in both wild type and mGlu3$^{-/-}$ mice at PND30. Reduction of BDNF in response to maternal stress has been reported several times strengthening the validation of our MRS animal model (Boersma et al., 2014; Fatima et al., 2019).

The brain is astonishing plastic during early post-natal development (Kolb and Gibb, 2011). Several key events shape GABAergic system occur during this critical time window. For example, GABA shifts progressively from excitatory to inhibitory (Ben-Ari et al., 1989; Rivera et al., 1999; Ben-Ari et al., 2012), and formation of perineural nets surrounding GABAergic interneurons is associated with the closure of critical windows of cortical plasticity(Bartus et al., 2012). Hence, it is not so surprising to see that transcripts encoding for GABAergic interneuron-related genes vary during different time window of development. Consistently with this idea, we showed that different transcripts of epigenetic markers such as Mecp2, DNMT1 and HDAC3 were differentially expressed depending of the post-natal day. Indeed, DNMT1 mRNA levels were higher in wild type MRS mice and mGlu3$^{-/-}$ non-MRS mice, whereas DNMT1 mRNA levels were decreased by maternal stress at PND21 and PND30.

One of the most striking results observed in this study concerned the interplay between mGlu3 receptors and MRS. MRS in mGlu3$^{-/-}$ mice reversed some alterations in gene expression induced by the deletion of mGlu3 receptors. This was prominent at PND21 when transcript levels of GABAergic interneuron-related proteins as parvalbumin, Sst, reelin, calretinin and GAT1 in MRS in mGlu3$^{-/-}$ mice were similar to wild type non-MRS mice. Interestingly, we observed a similar profile for GAD65 at PND30. Depending of the type of stress and temporal window of exposure, maternal stress can lead to adaptation or maladaptation. This idea is well represented here. Indeed, MRS in mGlu3$^{-/-}$ mice can also lead

to maladaptation as shown by PV transcript levels at PND9, which were overexpressed. Similar profiles were observed also at PND21 for NPY, GAD65. Interestingly, other studies reported that maternal stress, in addition to heterozygous deletion of Gad1, could specifically alter the proliferation of neurons committed to differentiate into PV-positive GABAergic interneurons (Wang et al., 2018; Uchida et al., 2014). This supports the hypothesis that both environmental and genetic factors shape the development of the GABAergic system in the hippocampus.

In summary, our findings demonstrate that the deletion of mGlu3 receptors and MRS alter the expression of genes related to GABAergic interneurons during the first 30 days of post-natal life. In addition, these changes were associated with different gene expression of stress markers and epigenetic marks that are usually observed animal models of early life stress (Matrisciano et al., 2012; Matrisciano et al., 2013). Furthermore, the interaction between the deletion of mGlu3 receptors and MRS seems to have compensatory and protective effects on the expression of several GABAergic interneuron-related genes. The prominent role of mGlu3 receptors on GABAergic neurons is not only observed in the prefrontal cortex but also in the hippocampus, a brain region that represents a key target of early life stress. Finally, some of these changes are relevant for human neuropsychiatric disorders such as schizophrenia. Indeed, a growing body of evidence indicates that the GABAergic system is altered in schizophrenia as demonstrated by post-mortem and neuroimaging studies (Glausier and Lewis 2017; Gonzales-Burgos et al., 2011; Nakazawa et al., 2012). All together these results paw the way for new therapeutic approach involving mGlu3 receptors as a main target.

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GENERAL DISCUSSION

Genes and environmental factors shape brain development and behavior. Stressful events occurring during perinatal life (e.g. prenatal and early post-natal life) contribute to program the developmental trajectory of the offspring. Using the perinatal stress (PRS) model in rats (*Maccari et al., 1995*) we have shown that PRS triggers long-lasting effects on the glutamatergic synapse across several brain regions and on the dopaminergic nigro-striatal system. Glutamatergic system impairment lies at the core of the PRS phenotype. Because mGlu2/3 receptors were constantly decreased by PRS across lifespan we further investigated the role of mGlu2 and mGlu3 receptors on brain development. We could demonstrate a central role of mGlu3 receptors in the developmental trajectory of cortical GABAergic system. By submitting mGlu3$^{-/-}$ to a maternal restraint stress (MRS), we showed the important role of the interplay between mGlu3 receptors and MRS on GABAergic system development in the hippocampus.

1) Alteration of the glutamatergic synapse lies at the core of PRS phenotype across the lifespan

Hypofunction of the glutamatergic synapse in the ventral hippocampus lies at the core of the phenotype triggered by PRS (*Marrocco et al., 2012*; *Marrocco et al., 2014*). Our results strengthen this idea since we observed also a hypofunction of the glutamatergic synapse in the ventral hippocampus but also in the dorsal hippocampus and prefrontal cortex in aged male PRS rats. This hypofunction was notably characterized by a global decrease of metabotropic and ionotropic glutamate receptors. The most striking results observed were the decrease of mGlu5 receptors in males across several brain regions (e.g. ventral hippocampus, dorsal hippocampus and prefrontal cortex) and the reduction of mGlu2/3 receptors by PRS in both sexes in ventral hippocampus and prefrontal cortex. Same results have been described previously in pups (*Laloux et al., 2012*) and adult rats (*Zuena et al., 2008*). Long-lasting effects of PRS were not only found on glutamate related proteins but also at a behavioral level. Indeed, we observed the same sex-dimorphic effect induced by PRS on the risk-taking behavior in the elevated plus maze in aged animals and in adult animals (*Zuena et al., 2008*). Furthermore, it is important to note that a causal relationship between risk-taking behavior and reduction in glutamate release has been demonstrated (*Marrocco et al., 2012*).

Interestingly, activation of the mGlu2/3 receptors with the agonist, LY354740, induces an increased risk-taking behavior in male rats (*Schoepp et al., 2003*; *Linden et al., 2005*), and mGlu2/3 receptors are targets for anxiety and stress disorders in humans (*Swanson et al., 2005; Tornese et al., 2019*; *Duman et al., 2019*). Although our founding highlights the important role of glutamate in early life stress, they also raise questions, in particular about sex-dimorphic effects induced by PRS.

2) Perinatal stress induces sex-dimorphic effects during aging

In our study, we have shown that PRS differentially impacts males and females during aging, in particular by inducing a dysmasculinization of the glutamatergic synapse in ventral and dorsal hippocampus as well as prefrontal cortex, that are known to be brain regions sensitive to stress (*McEwen et al., 2016*). These results were reinforced with the observation that PRS decreases testosterone levels in plasma and induced sex-dimorphic effects on estradiol levels and aromatase protein levels. Similar pattern of dysmasculinization have been reported concerning measures of stress responsivity (*Mueller and Bale, 2007, 2008)* as well as at a physiological, and behavioral level *(Ward, 1972, 1984*; *Becker and Kowall, 1977*; *Dahlöf et al., 1977*; *Biala et al., 2010).* Since brain organizes in a sexually dimorphic manner after a surge of gonadal hormone during the perinatal sensitive period (*McCarthy, 1994*; *McCarthy, 2016*), we can hypothetize that PRS alters the developmental trajectory of the sex-dimorphic brain. This is supported by previously study showing that PRS decrease plasma testosterone levels during the fetal life (*Ward et al., 1972*). Moreover, a reduction of aromatase activity (the enzyme converting testosterone into estradiol) in hypothalamic and amygdaloid homogenates of male and female rat fetuses has been reported (*Weisz et al., 1982)*. Furthermore, aromatase inhibition in neonatal brain, dramatically dysmasculinizes the neonatal brain miRNA environment during the perinatal sensitive period (*Morgan and Bale, 2011*). Because aromatase seems to play a central role in the dysmasculinization effects triggers by early life stress, we can think that the enzyme could be involved in the dysmasculinization profile of the glutamatergic synapse that was observed in aged males PRS. Since glucocorticoids mediate the prenatal programming of the HPA axis (*Barbazanges et al., 1996*), it is reasonable to suppose that elevation of glucocorticoids following perinatal stress (*Maccari et al., 1995*) alter aromatase activity during perinatal period resulting in changes in gonadal hormones during puberty. Several evidences suggest that developmental programming of adult hippocampal function relies on the central role of androgens as well as their aromatized by product, estrogens (*Kight and McCarthy, 2020*). Furthermore, estrogens

and androgens as well as glucocorticoids influence the glutamatergic transmission (*Foradori et al., 2007*; *Farkas et al., 2018; Popoli et, al., 2011*). Because, glutamatergic hypofunction in the ventral hippocampus lies at the core of the PRS phenotype, we can hypothesize that elevation of glucocorticoids levels and changes in hormonal levels during critical period act together to program the dysmasculinization of the glutamatergic synapse that we observed during adulthood until aging.

While PRS led to a hypofunction of the glutamatergic synapse in males, PRS had just few effects on females and even had positive effects on other neurobiological parameters. PRS, in females, increased the risk-taking behavior, BDNF protein levels, oxytocin receptors and reduced interleukin-6 levels, a cytokine pro-inflammatory suggesting that females are protected against PRS. Although these results prompt for taking account both sexes in early life stress studies, they also question about the programming effect of PRS. Different hypothesis has been formulated concerning the (mal)adaptation following early life stress.

The "cumulative stress hypothesis" states that repeated exposure to stressful events is the main environmental factor for pathological onset (*McEwen BS, 2000; McEwen 2008)*. Hence, recurrent adverse events, especially when added to perinatal stress, exacerbate psychopathological conditions. On the other side, according to the "match/mismatch hypothesis," early-life stress could also be somehow protective against stressors in late life, leading to better achievement of adaptation and survival (*Daskalakis et al., 2013; Schmidt, 2011; Belsky and Pluess, 2009)*. Consistently with the mismatch hypothesis, females PRS submitted to intense stress in middle age show a more dynamic phenotype than non-stressed individuals (*Van-Camp et al., 2018*). Hence, it raises the attractive hypothesis that females PRS answer to the match/mismatch hypothesis whereas male, that are more sensitive to PRS, respond to the "cumulative stress hypothesis".

We demonstrated that males during aging are more sensitive than females to PRS; hence we continued our project focusing on long-term effects of PRS in males.

3) Perinatal stress programs an accelerated aging of the basal ganglia motor system

Motor performance declines during aging including increased variability of movement (*Contreras-Vidal et al., 1998*; *Darling et al., 1989*), slowing of movement (*Diggles-Buckles, 1993*), coordination difficulty (*Seidler et al., 2002*) and difficulties with balance and gait (*Tang & Woollacott, 1997*). Age-correlated changes in neurotransmitter systems of the basal ganglia have been postulated to contribute to the disruption of motor function and balance

associated with aging (*McNeill et al., 1984*). In our study, we could demonstrate that perinatal stress programs an accelerated aging of the basal ganglia motor system and related motor behavior. In this regard, we showed that adult PRS rats displayed poor performance in a battery of behavioral tests that require a correct functioning of the striatal motor programming. Motor impairments were associated with neurochemical changes found in the striatum of adult PRS rats are suggestive of an impaired nigrostriatal dopaminergic transmission. We showed that PRS in adult rats increased activity of the indirect pathway as showed by increased A_{2A} receptor signaling and deceased D_2 receptors signaling and a reduction of dopaminergic release in striatum. Furthermore, by studying behavior and nigrostriatal system in both adult and aged rats we could observe that PRS decreased dopaminergic release in striatum and reduced motor behaviors. Hence, these results highlight the long-lasting effects of PRS programming effects concordantly with the work presented previously. Another very interesting point from this work was that PRS adult rats displayed similar neurochemical and behavioral pattern compared to unstressed old rats. Thus, it suggests that PRS programs, in adult rats, an accelerated aging of the basal ganglia motor system by lowering the age-dependent threshold for motor dysfunction.

The hypothesis that PRS programs an accelerating aging comes into resonance with our previous results showing a hypofunction of the glutamatergic synapse in aged male PRS. Whether or not glutamate release decrease with aging is not so clear. Studies aiming to answer to this question reported a decrease of glutamate release (*Zhang et al., 1991*; *Minkeviciene et al., 2008*) with aging, no change (*Dawson et al., 1989*; *Meldrum et al., 1992*) or an increase (*Massieu and Tapia, 1997*; *Saransaari and Oja, 1995*) in cerebral cortex and hippocampus. The most consistent findings in relation to glutamate and aging of the brain are the reduction of NMDA receptors density in most cortical area, hippocampus and striatum has been described consistently (*Nicolle et al., 1996*; *Magnusson et al., 2012*). The decrease of NMDA receptors has notably been observed in aged PRS animals in ventral hippocampus and prefrontal cortex. In the chapter I, we also showed that PRS reduces synaptic vesicle-associated proteins that are involved in neurotransmitters release (*Sudholf, 2012*). Interestingly, reduction in expression of neurotransmission-regulating proteins with aging has been reported (*Vanguilder et al., 2011*). As for glutamatergic neurotransmission, healthy aging is associated with prominent declines in multiple components of the brain's dopamine system (*Wang et al., 1998*; *Bäckman et al., 2000*; *Mozley et al., 2001*; *Erixon-Lindroth et al., 2005*). Among the dopaminergic markers changed by PRS, the decrease of D_1 receptors with aging has been also reported in human (*Wang et al., 1998*). Additionally to the impairment of

glutamatergic and dopaminergic neurotransmission, PRS impairs aging-related process such as the inhibition of neurogenesis in the hippocampus (*Lemaire et al., 2000*), a pro-inflammatory profile (*Vanbesien-Mailliot et al., 2007*); a disruption of estrous cycle in adult (*Reynaert et al., 2016*) and middle aged females (*Van-Camp et al., 2018*) and a prolongation of stress-induced corticosterone secretion (*Maccari et al., 1995*). Taken together, these data suggest that PRS lead to a global aging acceleration process.

4) mGlu3 receptors is important for cortical GABAergic system development

Glutamatergic system plays a central role in the PRS phenotype. Because mGlu2/3 receptors were constantly decreased by PRS across lifespan (*Laloux et al., 2012*; *Zuena et al., 2008*) until aging (chapter one), we further investigated the role of mGlu2 and mGlu3 receptors on brain development. We could demonstrate a central role of mGlu3 receptors in the developmental trajectory of cortical GABAergic system. The deletion of mGlu3 receptors impairs the following key events that shape, the developmental trajectory of inhibitory GABAergic transmission in the cerebral cortex: 1) postnatal maturation of GABAergic interneurons, which acquire specific peptide-protein markers; 2) the GABA shift (i.e., the developmental changes in neuronal chloride homeostasis driven by KCC2 (*Rivera et al., 1999*; *Ben Ari 2012*); 3) the formation of PNNs, which restrain the plasticity of PV^+ interneurons and other neuronal types (*Uena et al., 2017*) and 4) potentially the correct matching between pyramidal neurons and GABAergic interneurons (*Wong et al., 2018*). We showed a constant decrease of parvalbumin proteins levels and NMDA receptors during development and in adult. NMDA receptors are highly expressed and constitutively active in PV^+ cells and other interneurons (*Moghaddam et al., 2012*). In this regard, NMDA receptors in parvalbumin interneurons are critical for normal gamma rhythms and specific cognitive behavior (*Carlen et al., 2012*). The role of NMDA receptors expressed on PV^+ interneurons in gamma rhythms is beginning to be unraveled. Recent study suggests that NMDA receptors activation enhances PV^+ interneurons to pyramidal neurons inhibition in a manner consistent with presynaptic mechanisms in prefrontal cortex (*Pafundo et al., 2018*). Parvalbumin interneurons are enwrapped by perineural nets (PNN), extracellular matrix structures involved in synaptic plasticity (*Favuzzi et al., 2017*; *Wang et al., 2012*). We demonstrated that PNN enwrapping PV^+ neurons are increased during development. Because condensed PNN is thought to act as a molecular brake on synaptic plasticity as critical periods close (*Hockfield et al., 1990*), it is possible that the deletion of mGlu3 receptors restrain the plasticity of PV^+

neurons by closing the critical period sooner. Hence, these data suggest a central role for mGlu3 receptors in parvalbumin interneurons development.

Additionally, the deletion of mGlu3 receptors decreased KCC2 expression, a chloride potassium symporter and depolarized the reversal potential of GABA at PND9. Since the GABA shift from excitatory to inhibitory depends of KCC2 expression during early development (*Rivera et al., 1999*), these data suggest that mGlu3 receptor is involved in the GABA shift, maybe by acting on KCC2 expression by a mechanism that remains to be determined. Furthermore, we demonstrated that the deletion of mGlu3 receptor reduces global oscillatory activity in all frequencies. Distinct subtypes of inhibitory interneuron are known to shape diverse rhythmic activities in the cortex. For exemple, PV^+ neurons are involved in generating gamma (γ) oscillations (from 35 to 45 Hz) in local field potentials (*Feuchs et al., 2007*; *Sohal et al., 2009*) whereas somatostatin interneurons activity correlates with beta (β) oscillations (*Hu et al., 2011*) although they interact together to orchestrate specific band activity (*Chen et al., 2017*). Thus, the present findings demonstrate that mGlu3 receptors are required for a proper development of GABAergic neurotransmission in the PFC and suggest that a dysfunction of these receptors alters the developmental trajectory of cortical GABAergic interneurons.

Most of the changes induced by the deletion of mGlu3 receptors on the cortical GABAergic system development could be reminiscent of those found in schizophrenic patients. Indeed, growing body of evidence indicates that the GABAergic system is altered in schizophrenic treated patients as demonstrated by post-mortem and neuroimaging studies (*Glausier and Lewis 2017*; *Gonzales-Burgos et al., 2011*; *Nakazawa et al., 2012*). In particular, parvalbumin interneurons may play a central role in GABAergic system alteration in schizophrenia (*Enwright et al., 2018*; *Hashimoto et al., 2003*) because they are involved in the alteration of cortical network oscillations found in schizophrenia (*Gonzales-Burgos et al., 2015*). In addition, GABAergic system impairment in prefrontal cortex may cause cognitive alteration observed in schizophrenic treated patients (*Xu and Wong, 2018*). All together these results paw the way for new therapeutic approach involving mGlu3 receptors as a potential target for cognitive symptoms in schizophrenia.

5) <u>mGlu3 receptors and maternal restraint stress influence together neuroplasticity genes expression in hippocampus during development</u>

By submitting wild-type mice and $mGlu3^{-/-}$ mice to a maternal restraint stress (MRS) during a critical period for GABAergic progenitors migration and maturation, we strengthened the

important role of mGlu3 receptors and the influence of the interplay between mGlu3 receptors and MRS on GABAergic system development in the hippocampus. Indeed, we still observed a significant reduction of parvalbumin transcripts in mGlu3$^{-/-}$ mice at PND21 and a large trend to a decrease at PND30. Furthermore, the deletion of mGlu3 receptors massively decreased other transcripts encoding for GABAergic related genes such as calretinin, neuropeptide Y, and GAD67 at PND21 in the hippocampus. Hence, it suggests that the important role of mGlu3 receptors on GABAergic system development is not limited to the neocortex. MRS strongly reduced GABAergic markers in the hippocampus at PND30 consistently with previous studies suggesting that prenatal stress alters GABAergic interneurons development (*Lussier and Stevens, 2016*; *Stevens et al., 2013*). Very interestingly, we could observe a decrease of glucocorticoids receptors (GRs) transcripts in MRS rats. Since a reduction of GR has been found in PRS rats and is one of the reason of the elevation of glucocorticoids (*Maccari et al., 1995*), it is reasonable to think that a short period of intense stress during prenatal life is sufficient to program stress response maladaptation. Consistently with this idea, we observed several changes of epigenetic markers that are known to lie at the core of the programming effects triggers by PRS (*Vaiserman, 2015*; *Maccari et al., 2014*). This study revealed also that interplay between mGlu3 receptors and MRS lead to adaptation or maladaptation. The adaptation induced by the interplay is particularly noticeable at PND21 where, mGlu3$^{-/-}$ mice submitted to MRS showed similar transcript levels of parvalbumin, somatostatin, calretinin, GAT1 and reelin were similar to unstressed wild type mice. At the opposite, MRS in mGlu3$^{-/-}$ mice induced an overexpression of parvalbumin transcripts at PND9. These results highlight the influence of the interaction between genes and environment in brain development.

Conclusions

In summary, we could demonstrate that 1) long-term programming triggers by PRS is strictly sex-dependent; 2) PRS induces a dysmasculinization of the glutamatergic synapse in ventral hippocampus, dorsal hippocampus and prefrontal cortex; 3) females, during aging, are protected against PRS; 4) PRS programs an accelerated aging of the basal ganglia motor system; 5) mGlu3 receptors shape the developmental trajectory of cortical GABAergic system and 6) mGlu3 receptors and MRS influence together neuroplasticity genes expression in hippocampus during development.

Perspectives

Although these studies shed new lights on the role of perinatal stress and metabotropic glutamatergic in the developmental trajectory of the central nervous system, they raise also new questions. Women and men are different but in the same time equals because they belong to the same species. In biology, all cells have a sex, designated by the presence of X or Y chromosomes, which in most cases will be XX (female) or XY (male), however, this alone cannot explain the multiple differences between women and men. Understanding how sex-differences shape the balance between vulnerability and resilience to stress is becoming critical.

The work of my double PhD thesis has permitted to show that long-term programming triggers by PRS is strictly sex-dependent. Consequently, it is necessary to investigate in depth the potential mechanisms leading to distinct profile between male and females PRS. Since we observed a dysmasculinization of the glutamatergic synapse in PRS males it is reasonable to suppose that alteration of the aromatase by PRS during critical period could be one of the reasons. We also need to understand why females are less vulnerable to PRS than males during aging. Males and females differ by gonadal hormones. In the light of the protective effects of estrogens reported (*McEwen and Milner, 2017*; *Zárate et al., 2017*) it should be interesting to investigate whether or not estrogens are responsible of the protective effects display by females.

Because PRS programs an accelerated aging of the basal ganglia motor system it is possible that stressful events occurring during early life increase the risk to develop aging related neurological disorders. Stress related disorders such as depression are found in comorbidity with Parkinson's disease and furthermore, depression is a common non-motor symptom preceding motor symptom in PD (*Dallé and Mabandla, 2018*). Hence, investigating a potential role of early life stress on Parkinson's disease is crucial. To this purpose, it could be proposed two research approaches: 1) in clinic, retrospective studies under the supervision of expert neuropharmacologists in Parkinson's disease and 2) in preclinic researches. Concerning preclinic research, a starting point could be to induce 6-OHDA lesions in PRS rats, both females and males, in order to see rather or not PRS worsen the neurobiological and behavioral changes induced by 6-OHDA lesions. Considering that our results suggest that, in male, PRS lead to a global accelerating aging of the organism it could be very interesting to test this hypothesis by analyzing hallmarks of aging. These hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-

sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication *(López-Otín et al., 2013*).

Although we demonstrated that mGlu3 is involved in the cortical GABAergic system development it remains to be determined by which mechanism these changes occur. mGlu3 is expressed in all tripartite synapse compartment, hence several mechanisms could be proposed. Alterations in glutamatergic transmission onto developing GABAergic systems, in particular onto parvalbumin-positive PV^+ fast-spiking interneurons, have been proposed as underlying causes of several neurodevelopmental disorders, including schizophrenia and autism. In this regard, it would essential to test whether mGlu3 receptors expressed in parvalbumin interneurons are involved in the GABAergic developmental abnormalities described previously. Genetic strategies deleting mGlu3 receptors specifically in parvalbumin interneurons could be used.

Interplay between mGlu3 receptors and MRS influence the development of neuroplasticity gene expressions in hippocampus. Further studies are needed to investigate the impact of this interplay on other brain regions and on the functionality of the GABAergic system and related behavior such as working memory.

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Abstract

Stressful events occurring during perinatal life programs the emergence of pathological phenotypes later in life. By using the perinatal stress rat model, we investigated the long-lasting effects of perinatal stress (PRS) on the glutamatergic synapse in males and females. Remarkably, we demonstrated that long-term programming of PRS is strictly sex-dependent and induces a dysmasculinization of the glutamatergic synapse whereas old females PRS rats were protected. Because motor functions decrease during aging, we further investigated the long-term effects of PRS on the basal ganglia, a group of subcortical nuclei involved in motor functions. We could demonstrate that perinatal stress programs an accelerated aging of the basal ganglia motor system. Since metabotropic glutamate receptors 2/3 (mGlu2/3) are constantly decreased by PRS across lifespan in both sexes, we studied the role of mGlu2 and mGlu3 in brain development. We showed that mGlu3 receptors shape the developmental trajectory of cortical GABAergic system. Considering our findings showing that mGlu3 receptors and early life stress influence the brain development, we investigated how the interplay between these environmental and genetic factors impact neuroplasticity markers during development in hippocampus, a central region in stress response. We observed that mGlu3 and maternal restraint stress (MRS) alter GABAergic interneurons related genes expression, stress and epigenetic markers. Surprisingly, mice lacking mGlu3 receptors submitted to MRS showed compensatory mechanisms during specific time window during development. Taken together our results strengthen the idea that nature and nurture shape brain development.

Résumé

Les événements stressants survenant au cours de la vie périnatale programment l'émergence de maladies au cours de la vie. En utilisant le modèle de stress périnatal chez le rat, nous avons étudié les effets à long terme du stress périnatal (PRS) sur la synapse glutamatergique chez les mâles et les femelles. Remarquablement, nous avons démontré que la programmation à long terme du PRS est strictement dépendante du sexe et induit une démasculinisation de la synapse glutamatergique alors que les femelles PRS âgés étaient protégées. Parce que les fonctions motrices diminuent au cours du vieillissement, nous avons approfondi les effets à long terme du PRS sur les ganglions de la base, un groupe de noyaux sous-corticaux impliqués dans les fonctions motrices. Ainsi, nous avons pu démontrer qu'un stress périnatal induit un vieillissement accéléré du système moteur des ganglions de la base. Étant donné que les récepteurs métabotropiques au glutamate 2/3 (mGlu2/3) sont constamment diminués par le PRS chez les mâles et femelles au cours de la vie, nous avons étudié le rôle des récepteurs mGlu2 et mGlu3 dans le développement du cerveau. Nous avons montré que les récepteurs mGlu3 façonnent la trajectoire développementale du système GABAergique cortical. Compte tenu de nos résultats montrant que les récepteurs mGlu3 et le stress précoce influencent le développement du cerveau, nous avons étudié l'influence de l'interaction entre ces facteurs environnementaux et génétiques sur les marqueurs de la neuroplasticité lors du développement de l'hippocampe, une région centrale de la réponse au stress. Nous avons observé que les récepteurs mGlu3 et le stress précoce (MRS) altèrent l'expression des gènes liés aux interneurones GABAergiques ainsi que les marqueurs du stress et les marqueurs épigénétiques. Étonnamment, les souris dépourvues de récepteurs mGlu3 soumis au MRS ont montré des mécanismes compensatoires pendant une fenêtre de temps spécifique au cours du développement. Ensemble, nos résultats renforcent l'idée que les gènes et l'environnement façonnent le développement du cerveau.