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Stress exposure across lifespan shapes the CNS susceptibility to further adverse events: preclinical evidence using a double hit approach

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Stress exposure is known not only to be a risk factor for the development of psychiatric disorders itself, but also to exacerbate other medical conditions, prompting structural and functional changes that can eventually evolve from a normal adaptive body reaction to a pathological state (Gradus, 2017).

On this basis, the aim of my PhD project was to investigate at preclinical level how and by which molecular mechanisms stress exposure can leave a signature in the individual, thus increasing the susceptibility of the central nervous system (CNS) to stress-related disorders or aggravating the outcome of illnesses occurring later in life. To achieve this goal, I took advantage of a "double hit" approach that implies that a 'first hit', mostly during critical periods of development, disrupts the ontogeny of neural systems and establishes a vulnerability to a 'second hit' later in life. Among the multiple mechanisms involved in stress-susceptibility, I have focused my analyses on neuroplasticity, neuroinflammation and oxidative damage. Moreover, considering that one of the most crucial variables of stress response extent is when the individual experiences it, I have investigated the long-lasting impact of stress occurring in different time windows.

Specifically, I firstly focused on the gestational period, a so-called "window of vulnerability" (Briscoe et al., 2016) since the exposure to adverse events during pregnancy has been shown to impact not only on maternal health but also to have a deep long-lasting influence on the offspring neurodevelopment, leading to enhanced susceptibility to diseases and dysfunctions during adulthood (Zucchi et al., 2013; Coe and Lubach, 2005; Entringer et al., 2015). Therefore, in the first part of my project I examined the long-term effects of stress occurring during the intrauterine life on the clinical manifestations of a well-established animal model of multiple sclerosis, the Experimental Autoimmune Encephalomyelitis (EAE), a chronic and inflammatory condition characterized by loss of myelin (Robinson et al., 2014). My results

demonstrated that gestational stress induced a marked increase in the severity of EAE symptoms in the adult mouse. Further, I highlighted an altered maturation of oligodendrocytes in the spinal cord of prenatally stressed EAE animals. These behavioral and molecular alterations were paralleled by changes in the expression and signaling of the neurotrophin BDNF, an important mediator of neural plasticity that may contribute to stress-induced impaired remyelination (Murray and Holmes, 2011).

Furthermore, since patients affected by stress-related disorders present deficit in the neuroplastic mechanisms that are normally set in motion in response to external challenging stimuli (Wang et al., 2017), I investigated the influence of stress *in utero* on the response to a second challenge in adulthood, exposing prenatally stressed mice to a further acute stress. The molecular analyses in the hippocampus revealed that fetal stress resulted not only in increased activation of the immune system itself, but also in an impairment of the proper responsiveness of the redox machinery to the second stress.

I then focused on adolescence, a sensitive period for brain development and thus also for environmental stimuli including stress. Social stress, such as bullying or subordination, is among the most prevalent stressors throughout adolescence and is strongly related to an enhanced susceptibility to diseases and dysfunctions later in adulthood (Lupien et al., 2009). Moreover, adolescents are more prone to brain concussion, and indeed 15/19-year age juvenile are experiencing the highest rates of incidence of traumatic brain injury (TBI) (Kimbler et al., 2011). As such, I spent 6 months as a visiting PhD student at the laboratory of Brain Injury, Neuroinflammation and Cognitive Function headed by Professor Susanna Rosi at the University of California, San Francisco, to investigate if and how exposure to social stress during adolescence can alter the TBI outcome. Specifically, in this part of the PhD project, adolescent mice were exposed to the social defeat stress

protocol before being injured with a new model of TBI, the repetitive closed-head impact model of engineered rotational acceleration (CHIMERA). The results highlighted that stressed mice developed anxiety-like features, regardless the concussions, while stress and brain injury have a reciprocal influence in the NOR test, where only mice that were both stressed and exposed to TBI did not display impairment in the ability to recognize the novel object. Paralleled to these behavioral effects, we didn't find differences in hippocampal microglia activation.
Lastly, I have investigated stress exposure during adulthood, focusing on the potential long-lasting impact of a chronic stress paradigm -known to induce psychiatric-like phenotype in preclinical model- in altering the responsiveness to a second acute challenge after a recovery period of 3 weeks. The molecular analyses, focused on modulators of the oxidative balance, demonstrated that the second hit was able to strongly induce the gene expression of Sulfiredoxin 1 (Srxn1) and Metallothionein-1a (Mt-1a), two antioxidant genes. This beneficial effect set in motion to cope with the sudden challenging situation was impaired by the previous exposure to chronic stress. Interestingly, chronic treatment with the antipsychotic lurasidone partially restored the appropriate acute responsiveness.

The results obtained during my PhD project by using the double hit approach in different periods of life, indicate neuroinflammation, altered oxidative balance and impaired neuroplasticity as common molecular targets underlying the impact of a previous stress in shaping the vulnerability to further adverse conditions, providing new information on the etiopathogenesis of stress-related diseases.

“Ogni stress lascia un’indelebile cicatrice, l’organismo paga questo diventando un po’ più vecchio dopo una situazione di stress” H.Selye, 1956

Vivere esperienze stressanti, anche apparentemente innocue, può incidere fortemente sul nostro equilibrio omeostatico e avere diverse conseguenze patologiche, nell’eventualità il nostro corpo non reagisca in maniera fisiologica e adattativa. Lo stress è, infatti, un noto fattore di rischio per lo sviluppo di patologie psichiatriche e non.
A tal riguardo, lo scopo della mia tesi di dottorato è stato studiare come -e attraverso quali meccanismi molecolari- un evento stressante possa lasciare una “traccia” nell’individuo, predisponendolo così ad una maggiore suscettibilità verso condizioni patologiche che possono successivamente verificarsi. Le mie analisi si sono concentrate su alcuni noti bersagli molecolari dello stress, come ad esempio meccanismi di neuroplasticità, neuroinfiammazione e stress ossidativo. Inoltre, siccome l’età in cui si fa esperienza di forti stress è una tra le variabili che condizionano maggiormente le conseguenze dello stress stesso, ho focalizzato i miei studi su 3 diverse finestre temporali: il periodo perinatale, l’adolescenza e l’età adulta.
Nella prima parte del mio progetto ho approfondito il ruolo dello stress durante la gestazione (stress *in utero*), un periodo estremamente sensibile a stimoli esterni tanto da essere nominato “finestra di vulnerabilità”. Vi sono infatti diverse evidenze scientifiche che dimostrano come eventi avversi durante questa finestra temporale possano avere conseguenze di lunga durata, non solo sulla salute materna ma anche della progenie, aumentando così il rischio che i nascituri possano sviluppare disfunzioni patologiche una volta adulti. Più in dettaglio, ho studiato gli effetti a lungo termine dell’esposizione a stress prenatale sul decorso dell’encefalomielite autoimmune sperimentale (EAE), un modello preclinico avvalorato di sclerosi

multipla caratterizzato a sua volta da danno mielinico, che è stato indotto in topi da esperimento nati da madri stressate una volta adulti. I risultati ottenuti dimostrano che la precedente esposizione a stress peggiora significativamente la sintomatologia dell'encefalomielite. Abbiamo inoltre evidenziato una compromessa maturazione degli oligodendrociti a livello del midollo spinale degli animali precedentemente esposti a stress. Come possibile meccanismo sottostante abbiamo quindi ipotizzato che lo stress agisca sul signaling della neurotrofina BDNF, nota avere un ruolo neuroprotettivo, e di conseguenza comprometta il processo fisiologico di riparazione del danno mielinico.

Nella seconda parte del mio progetto ho invece analizzato un'altra potenziale conseguenza a lungo termine dello stress *in utero*, cioè come questo possa influenzare la capacità di risposta ad un secondo stress acuto. In individui sani, la normale risposta ad uno stress improvviso prevede la messa in modo di meccanismi adattativi basati sulle proprietà plastiche neuronali. Tuttavia, queste proprietà sono note essere alterate in soggetti affetti da malattie psichiatriche legate allo stress. Ho quindi sottoposto topi stressati prenatalmente ad un secondo stress acuto, più precisamente al paradigma di nuoto forzato. Le analisi condotte hanno poi evidenziato che la precedente esposizione a stress non solo modifica il grado d'infiammazione basale degli animali, ma per di più altera la normale responsività acuta di sistemi come quello del bilancio ossidativo che vengono fisiologicamente attivati in risposta a stress improvvisi.

Mi sono successivamente focalizzata sull'adolescenza, un altro periodo estremamente sensibile a stimoli ambientali come lo stress. Fenomeni di stress sociale come bullismo o subordinazione sono frequenti durante questa fascia d'età e possono predisporre gli adolescenti ad una maggiore vulnerabilità a sviluppare patologie e disfunzioni. Il terzo obiettivo della mia tesi è stato quindi valutare come stress sociali

durante questo periodo possano creare un substrato più sensibile ad altre esperienze molto frequenti durante l'adolescenza, come la concussione cerebrale a seguito di sport o incidenti. Ho lavorato a questo progetto presso il laboratorio di Brain Injury, Neuroinflammation and Cognitive Function, University of California, San Francisco sotto la supervisione della Professoressa Susanna Rosi, che studia diversi modelli di trauma cranico. Più nello specifico ho utilizzato il paradigma di social defeat stress come modello di stress sociale su topi adolescenti che successivamente hanno subito due concussioni di moderata entità, che ho indotto avvalendomi di un modello recentemente sviluppato di trauma (repetitive closed-head impact model of engineered rotational acceleration (CHIMERA). Ho poi analizzato l'attivazione di cellule microgliali, noti target sia dello stress che del trauma cranico, nell'ippocampo.
Infine, nella quarta sezione di questo lavoro ho utilizzato un modello di stress cronico da immobilizzazione per 4 settimane in ratti adulti. Questo paradigma di stress cronico è usato a livello sperimentale per indurre sintomi simili a quelli sviluppati da pazienti affetti da malattie psichiatriche legate allo stress. Per il mio progetto, ho esaminato come uno stress cronico in età adulta possa lasciare una "traccia", che rimane anche dopo 3 settimane di recupero quando invece i sintomi simil-psichiatrici si sono risolti, capace di alterare la risposta ad un secondo episodio acuto di immobilizzazione. Le mie analisi si sono concentrate su alterazioni a carico del sistema redox, il cui ruolo nell'eziologia delle malattie psichiatriche è ormai riconosciuto, e sulle proprietà terapeutiche e antiossidanti dell'antipsicotico lurasidone.

In conclusione, per questo progetto di tesi mi sono avvalsa di diversi approcci sperimentali per analizzare approfonditamente come uno stress, specialmente se vissuto in momenti sensibili della vita, possa rendere l'individuo più vulnerabile a successivi eventi avversi. I risultati

che ho ottenuto evidenziano come alterazioni a carico di sistemi di neuroplasticità, infiammazione e bilancio ossidativo possano essere meccanismi messi in moto dallo stress per lasciare questa “traccia” di suscettibilità.

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1. Introduction

1.1 Stress as environmental risk factor for different diseases

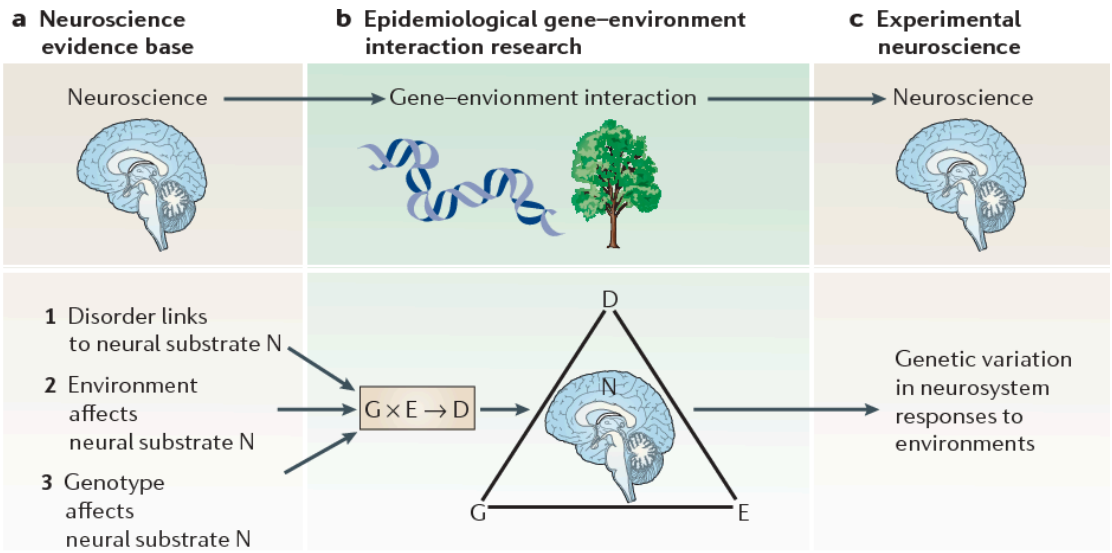
Environmental challenges are part of everyday life. Since most people are, at some point, exposed to physical or psychological stress events - such as loss of a dear one, violence, severe diseases diagnosis or natural disasters– carrying out adaptive mechanisms to face the stressful experience is a major priority. As a consequence, the body has developed an efficient set of connected biological systems to maintain homeostasis even in the most demanding situations. This “adaptive” machinery recruits several systems including, but not confined to, the autonomic nervous system (ANS), the hypothalamus pituitary adrenocortical axis (HPA), the metabolic and the immune system.

Hans Selye (1907–1983) is credited to be the first scientist who introduced the concept of stress in his theory of the “General Adaptation Syndrome” (1936). Selye postulated that stress response consists in a series of three stages: (1) alarm reaction, in which epinephrine and glucocorticoids are released to restore homeostasis; (2) resistance, in which defense and adaptation are sustained; and if the stress persists, (3) exhaustion, the consequence of which can be damaging. Selye’s theories have been reinterpreted over the years and the concept of “allostasis” has been introduced (McEwen, 1998). Allostasis is defined as the adaptive process by which stability and homeostasis are achieved, and the price the body has to pay to maintain this stability as a reaction to physical and psychological challenges is called “allostatic load”. While many mechanisms involved in allostasis are beneficial for the individual, others can render the organism more sensitive or susceptible to new challenges. Thus, when the cost to adaptation is excessive and exceeds the supplies, the body is facing an “allostatic overload” that can predispose individuals to illness. As a result, this

maladaptive response to stress has been strongly associated to a plethora of diverse diseases, some related to the psychiatric realm and others affecting different human body systems.

Trauma and stress-related psychiatric diseases -such as major depressive disorder (MDD), generalized anxiety disorder, post-traumatic stress disorder (PTSD), acute stress disorder and adjustment disorder- are a family of psychiatric diseases that arise following a stressful or traumatic event (Manual of Mental Disorders, DSM-5). Indeed, as other mental illnesses, stress-related mood disorders are known to result from a complex gene x environment interaction (GxE), in which the combination of a vulnerable genetic background and environmental risk factors -specifically stress- may contribute to their etiology (Sharma et al., 2016; Assary et al., 2018).

Furthermore, stress represents a risk and triggering factor for disorders that target different systems such as hypertension, ischemic heart diseases, heart failure, stroke and other cardiovascular diseases (Sara et al., 2018, Fioranelli et al., 2018), diseases of the gastrointestinal tract (Konturek et al., 2011; Bernstein, 2017), osteoporosis (Kelly et al., 2019), metabolic diseases such as diabetes (Harris et al., 2017; Kelly and Ismail, 2015), neurodegenerative diseases (Justice, 2018; Sotiropoulos, 2015) and demyelinating diseases such as Multiple Sclerosis (Briones-Buixassa et al., 2015; Riise et al., 2011).



Gene x Environment interaction (adapted from Caspi and Moffitt, 2006)

1.1.1 Stress response variability

As described above, the switch between allostatic load and overload is a complex and finely regulated process influenced by multiple factors. Some of these variables rely directly to the stressor itself (e.g., intensity, duration and type) and others are inherent to the individual (e.g., genetic background, biological age of exposure, social economic status and the capacity to cope with stress). As a result, mechanisms set in motion and final outcomes are strikingly distinct across individuals. Among the mentioned variables, in my PhD project I focused specifically on the time at which the stressful event occurs.

Preclinical and clinical studies have shown that early phases of life are particularly sensitive to stress, probably because the brain undergoes such important changes during these periods. In particular, the gestational period is a so-called “window of vulnerability” (Briscoe et al., 2016) and the exposure to adverse events during pregnancy has been shown to impact not only on maternal health but also to have a deep long-lasting influence on the offspring neurodevelopment, leading to an

enhanced susceptibility to diseases and dysfunctions during adulthood (Zucchi et al., 2013; Coe and Lubach, 2005; Entringer et al., 2015).
Adolescence is another vulnerable time of many psychosocial and physiological changes, including how an individual responds to stressors. Specifically, adolescence is marked by significant shifts in hypothalamic-pituitary-adrenal (HPA) axis reactivity, resulting in heightened stress-induced hormonal responses (Romeo, 2013).

Besides the evidences demonstrating the actual role of stress in the etiology and pathogenesis of such diseases, the underlying molecular mechanisms are still not elucidated. It is therefore relevant to investigate how and through which molecular systems and pathways the exposure to stressful experiences can influence so crucially several illnesses.

1.2 Molecular mechanisms of stress response

To understand how stress can contribute to illness development, it is primary to consider the mechanisms set in motion to adapt to stressful situations and to investigate how these initially positive and protective adjustments can become harmful for the body.

Hallmarks of stress response are the activation of the autonomic nervous system and of the hypothalamic-pituitary-adrenal (HPA) axis. In response to a stressor -paralleled by the activation of the sympathetic nervous system that affects several peripheral organs, including the adrenal medulla that releases epinephrine- the hypothalamus secretes the corticotropin-releasing hormone CRH, which induces the synthesis and release of the adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH then stimulates the release of corticosteroids (cortisol in human and corticosterone in rodents) that bind to their mineralocorticoid (MR) and glucocorticoid (GR) receptors and thus initiate/terminate the HPA axis stress response, via the negative feedback, and modulate acquisition processing, storage and retrieval of stressful experiences (Sapolsky et al., 2000). As a result, proper activation of the HPA-axis affects brain functioning to ensure adaptation to the stressor, but stress-induced mal-adaptation of the HPA-axis functionality may provide a mechanistic basis for stress-induced illness vulnerability.

Initiation of the stress response is not limited to activation of the ASN and HPA axis. Indeed, stress has been strongly associated with alterations of other mechanisms including neuroplasticity, immune response and redox homeostasis.

1.2.1 Neuroplasticity

The term neuroplasticity was used for the first time by Santiago Ramon y Cajal (1852-1934) (Ramón y Cajal, 1913-1914) and now referred to "the fundamental ability to make adaptive changes related to the structure and function of the nervous system" (Fuchs and Flugge, 2014). The molecular and cellular mechanisms underlying processes such as learning and memory are the best-characterized and most studied examples of neural plasticity. Stress is known to significantly influence learning and memory in a type, duration, and intensity of the stressor dependent way. It is generally assumed that short periods of stress can potentiate memory formation via synaptic plasticity -and this is likely to be the reason of long-term memories formation after traumatic events- whereas more severe or prolonged stressors can have deleterious effects upon learning and memory, leading even to amnesia, and upon several other aspects of cognition, both in human and in rodents (Joels et al., 2006; Shors, 2006; Anacker and Hen, 2017). Long-term potentiation (LTP) -an electrophysiological phenomenon of increase in synaptic strength produced by trains of stimuli- is one of the principal form of brain plasticity and one of the underlying processes of learning and memory. Over the past decades, it has been demonstrated that stress can lead to alterations of hippocampal LTP, following the inverted U-shaped dose effect fashion (Joels et al., 2006). Indeed, while exposure to stress-induced glucocorticoids in term of minutes to hours, such as what happens during an acute stress, is associated with enhanced LTP (Wang et al., 2019), prolonged periods of stress impair LTP functions (Joels and Krugers, 2007).

Among all the molecular regulators of neuronal plasticity, neurotrophic factors (NTFs), and in particular the neurotrophin family, are key players and are known to support survival, differentiation and maintenance of neuronal functions and finely modulate all the crucial steps of network

construction (Poo, 2001). The neurotrophin family comprises of nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) that have all evolved from a common neurotrophin ancestor gene. Their actions are dependent on binding to transmembrane receptor systems, the tropomyosin receptor tyrosine kinase family and the p75 neurotrophin receptor (Chao and Hempstead, 1995).

BDNF is a key mediator of activity-dependent processes and helps neurons to adapt and survive and speeds up the brain's ability to make new connections. The link between stress and BDNF is well recognized (McEwen et al., 2015) and to date, various animal models of stress have shown that negative stressors can affect neuroplasticity through altering Bdnf expression in the brain. For example, studies where rodents were exposed to early life stress, chronic stress or acute stress have significantly reduced Bdnf expression in different brain regions (Molteni et al., 2016; Luoni et al., 2016; Molteni et al., 2009; Bondar and Merkulova, 2016).

1.2.2 Neuroinflammation

Large body of evidence indicates that stress can activate inflammatory response both at peripheral and central level (Rohleder, 2014; Calcia et al., 2016). Both pro-inflammatory and anti-inflammatory mechanisms depend on the type, intensity and duration of stressors. Indeed, activation of the immune system is physiological when a stress is perceived, so that the body can be ready to react to possible injuries and infections subsequent to it, but can be detrimental when the stress is prolonged and mismanaged.

The immune system is composed of two interconnected branches. The first one is called innate immunity, is the body's first line of defence against tissue damage and microbial infection (Medzhitov, 2007). The effector cells of innate immunity constantly circulate in the body and

detect a wide variety of pathogens. These cells are able to signal the occurrence of injuries or infections and initiate a cascade of inflammatory processes that help contain an infection and promote healing and recovery (Medzhitov, 2007) When the innate immune defences are insufficient, these cells activate the second branch of the immune system, called adaptive immunity (Barton, 2008). In contrast to innate immunity, which is non-specific and does not confer long-lasting protection to the host, adaptive immunity involves the proliferation of specific white blood cells, such as lymphocytes, that attempt to neutralize or eliminate the intruders based on an immunological memory. Within the central nervous system (CNS), microglia cells are the key immune players and acquire a reactive profile to cope with altered homeostasis (Hanisch and Kettenmann, 2007), mainly by increasing the expression of pro-inflammatory mediators and neurotoxins (Reader et al., 2015; Ramirez et al., 2017).

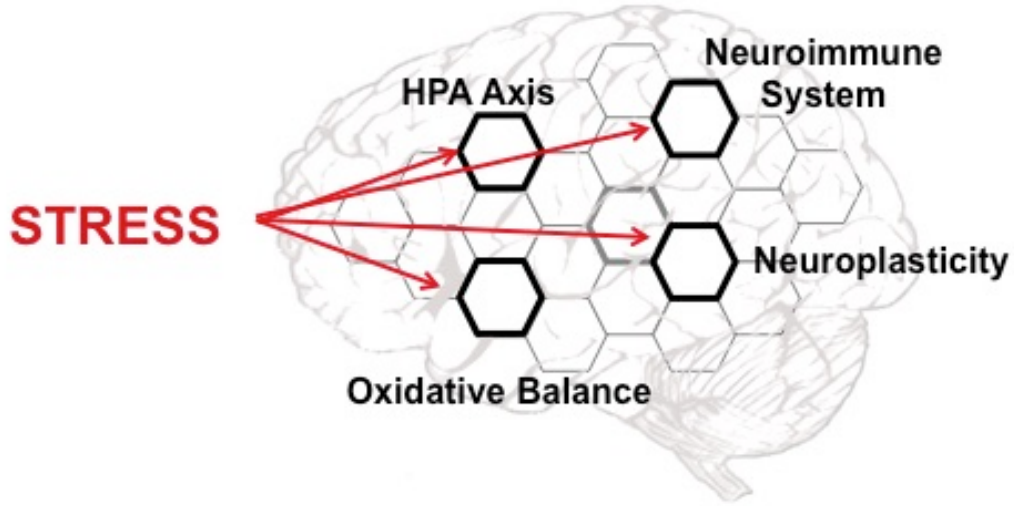
In the past years it has been shown that excessive inflammation directly contribute to pathophysiology of stress-related diseases. Indeed, preclinical models of stress paradigm known to induce psychiatric-like symptoms are associated with up-regulated expression of pro-inflammatory cytokines as well as microglia over-activation in stress-sensitive brain regions (Rossetti et al., 2016; Wang et al 2018; Hohmann et al., 2017, Weber et al., 2017, Stein et al., 2017).

1.2.3 Oxidative Stress

A further feature of stress response is the dysregulation of the redox balance and resultant oxidative stress. Oxidative stress -the imbalance between cellular production of reactive oxygen species and the counteracting antioxidant mechanisms- has been proposed to play several roles in the pathogenesis of chronic-degenerative conditions, such as athero-thrombotic events, neurodegeneration, cancer, some

forms of anemia, auto-immune diseases, and the entire comorbidity of uremia and diabetes (Galli et al., 2005).

Furthermore, several evidences link oxidative stress and mental disorders. One hypothesis for this association could be that the brain, besides it represents only a small portion of the total body weight, accounts for 20% of the overall energy expenditure in resting conditions and this high energy and oxygen demand, coupled with the unsaturated lipid enrichment, render it highly susceptible to redox unbalance (Cobley 2018). Interestingly, reactive oxygen species (ROS) are essential for neuronal signaling and function and are involved in several cellular processes, although they become detrimental when their production is excessive. Hence, the unbalance of the redox state due to increased production of ROS and/or the failure of antioxidant detoxifying mechanisms has been suggested to be a common pathogenic mechanism underlying many major psychiatric disorders. Evidences supporting this hypothesis come from clinical (Hassan et al., 2016; Salim, 2014; Ng et al., 2008; Schiavone et al., 2013) and preclinical studies. Specifically, using different experimental stress models, it has been demonstrated that paradigms such as chronic stress (Rossetti et al., 2018), acute stress (Casaril et al., 2019; Spiers et al., 2013), social defeat (Gao et al., 2019; Bouvier et al., 2017) or CORT treatment (Chen et al., 2019) can results in increased levels of pro-oxidant enzymes, lipid peroxidation and impairments of the antioxidant machinery.



Molecular targets of stress

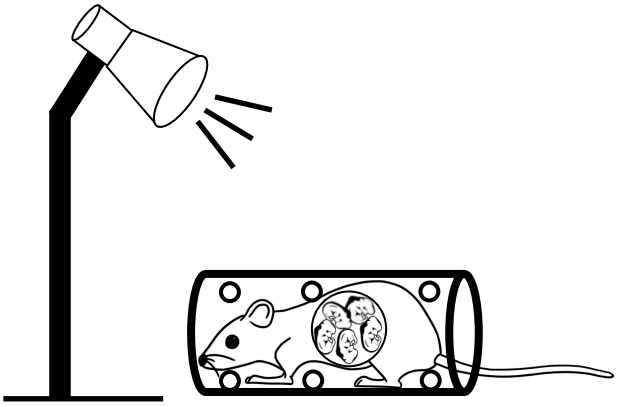
1.3 Stress-based experimental models

Despite animal models are not able to fully recapitulate human pathologies, especially considering the complexity, the multifaceted symptoms and the neurobiology of stress-related disorders, they represent a relevant tool for advancing our comprehension of the mechanisms underlying specific aspects of such diseases. In general, for the scientific community there are three main criteria for judging whether a particular model is properly useful to investigate a specific disease or condition: the *construct*, the *face* and the *predictive validity*. *Construct validity* refers to how well the mechanisms used to induce the disease phenotype in animals reflects the currently understood disease etiology in humans: the researchers should recreate in an animal the etiologic processes that cause a disease in humans and thus replicate the neural and behavioral features of the illness; *face validity* indicates that a model recapitulates important anatomical, biochemical, neuropathological, or behavioral features of a human disease whereas

predictive (or pharmacological) validity signifies that a model responds to treatments in a way that predicts the effects in humans.
In my project I took advantage of three well-established model of stress exposure: the prenatal stress, the social defeat stress and the chronic restraint stress.

1.3.1 Prenatal Stress

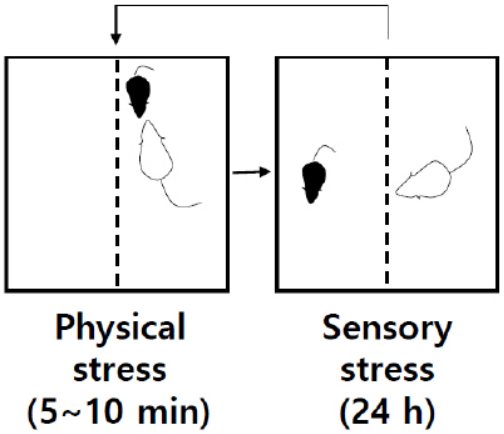
As mentioned before, the perinatal period is a critical time window when the so-called “fetal programming” occurs (Barker, 1998; Kwon and Kim, 2017). The theory of fetal programming proposes that the environment surrounding the fetus during its development plays a seminal role in determining its disease risk during the later stages. Indeed, in support of this, a growing amount of evidence from human and non-human studies shows that maternal stress during pregnancy exerts pervasive, long-lasting effects on the development of the fetal nervous system and, ultimately, on the offspring's physiology and behavior (Frasch et al., 2018). At molecular level, prenatal stress (PNS) has been associated with priming of the immune system and exacerbated response to inflammatory challenges in adulthood (Diz-Chaves et al., 2013) as well as impaired neural plasticity (Boersma et al., 2014). In preclinical studies, the most commonly used paradigm of prenatal stress consists in restraining pregnant dams in different phases of gestation with simultaneous exposure to a bright light (Weinstock, 2016).



Schematic representation of prenatal stress paradigm

1.3.2 Social Defeat Stress

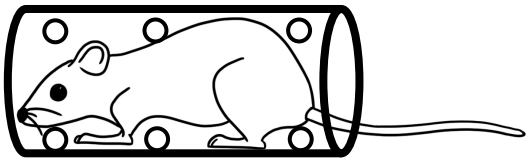
The social defeat paradigm uses social conflict between members of the same species to generate emotional and psychological stress and thus triggering depression-like behaviors -in particular social avoidance- as well as cognitive impairment (Hollis and Kabbaj, 2014; McKim et al., 2016). These behavioral derangements are associated with increased oxidative stress and inflammation (Patki et al., 2013) and impaired neuroplasticity (Blugeot et al., 2011). A widely used protocol of social defeat consists in introducing the experimental rodent into the home cage of an older, aggressive, dominant male. The intruder is quickly attacked and forced into subordination for the remaining of the physical interaction. The defeat experience, however, is not only a physical stressor. After a brief physical exposure and attack, intruders are often placed in a protective cage for the remaining of the test, allowing for psychogenic exposure to the resident without physical harm (Golden et al., 2011).



Schematic representation of social defeat paradigm

1.3.3 Chronic Restraint Stress

The use of restraint or immobilization for investigations of animal physiology, pathology and pharmacology has an extensive history (Paré and Glavin, 1986). Restraint is a preferred means of stressing animals, largely because it is straightforward, painless and without lasting debilitation. Indeed it doesn't involve any bodily harm to the animal subject once the period of the restraint is terminated. This ensures that any long-term effects of stress observed are due to the stressor that was applied, rather than to the physical repercussions of an irreversible or chronic injury. Immobilization can be acute or chronic, and with sessions lasting from 15/30 minutes to several hours (Paré and Glavin, 1986). Exposure to chronic restraint stress have been shown to induce depressive and anxiety-like phenotypes (Chiba et al., 2012) as well as cognitive deficits coupled by decreased BDNF levels in the hippocampus (Zhang et al., 2017), activation of the immune system at central level (Calcia et al., 2016) and oxidative stress (Salehi et al., 2018).



Schematic representation of a restrainer

2. Aim of the project

Stress is part of our everyday life. As a result, human natural selection has shaped a well and carefully orchestrated body response that relies on different system –including the autonomic nervous system and the HPA axis- to properly react to threats. An adequate response is therefore crucial to adapt to environmental changes that occur in different developmental stages throughout life. When the coping strategies set in motion after being exposed to a challenge are not sufficiently effective, the risk of maladaptive response increases. As a consequence, stress is known to be a crucial environmental component for the etiology of psychiatric diseases (Schmitt et al., 2014) but also to be an exacerbating risk factor for several diseases and conditions (Salleh, 2008).

With these premises, the overall goal of my PhD project was to deepen the mechanisms by which a previous stress exposure can prime and predispose the individual to be more vulnerable and prone to overreact when adverse conditions occur later in life. To achieve this result, my strategy has been to take advantage of different preclinical paradigms of stress and investigate how they create a “susceptible substrate” for further adverse challenges and diseases. Specifically, since time is one most crucial variable that influence stress response and consequences, I decided to focus on three specific time windows: early life, adolescence and adulthood. I then analyzed molecular systems known to be stress-sensitive-such as neuroplasticity, inflammatory activation and oxidative balance- in region of the CNS that are known to mediate molecular and behavioral features of stress response.

The first aim of the project was to evaluate the impact of a prenatal stress exposure on the susceptibility to the experimental autoimmune encephalomyelitis (EAE), a demyelinating condition of the CNS that resemble some feature of multiple sclerosis (MS). The contribution of environmental factors such as stress in prompting MS or influencing its

manifestations and course is not clearly elucidated (Heesen et al., 2007) and the few preclinical studies that have been carried out are focused on the long-term effects of neonatal manipulations (Krementsov and Teuscher, 2013; Teunis et al., 2002; Columba-Cabezas et al., 2009, Case et al., 2010). Therefore this study is -to the best of our knowledge- the first elucidating the impact of gestational stress. Our analyses were addressed to the neurotrophin BDNF, theorized to have a neuroprotective function in myelin repair, evaluating if prenatal stress affects myelination acting on this molecule and its signaling.

Secondly, since one of the features of stress-related disorders symptomatology is the impairment in neuroplastic mechanisms that are normally orchestrated to react to a new challenge or threat (Wang et al., 2017), the second objective of the project was to investigate the influence of stress *in utero* on the response to a second challenge in adulthood. More in detail, I exposed mice born from mothers stressed during pregnancy to a further acute stress, and investigate how the previous stress exposure influenced the physiological acute responsiveness of inflammatory markers and mediators of the oxidative balance.

Adolescence is known be another time window peculiarly sensitive to the environment (Jaworska and MacQueen, 2015). Specifically, psychosocial stress, such as bullying or subordination, is strongly related to an enhanced susceptibility to diseases and dysfunctions later in adulthood (Lupien et al., 2009). Therefore, the third aim of my project was to investigate how social stress can influence the final outcome of another high-incidence experience of adolescents, such as brain concussion. I worked on this project during the 6 months that I spent as a visiting PhD student at the laboratory of Brain Injury, Neuroinflammation and Cognitive Function headed by Professor Susanna Rosi at the University of California, San Francisco. More in detail, I exposed adolescent male mice to the social defeat stress

protocol and then gave them mild concussions using a recently developed experimental model of traumatic brain injury (TBI), the repetitive closed-head impact model of engineered rotational acceleration (CHIMERA). My analyses were then focused on the behavioral consequences of this combined condition and on the role of microglia, the resident immune cells of the brain, known to a be a key player in both stress and TBI-induced neuroinflammation.

To achieve the fourth and last objective of my project I took advantage of a well-established animal model of stress-related psychiatric diseases, the chronic restraint (CRS) paradigm, known to induce psychiatric-like phenotypes in experimental animals (Chiba et al., 2012). I examined how chronic stress during adulthood can leave a molecular signature that is still present, even after the animal recovers from the depressive-like behavioral derangements. To unmask such molecular signature, three weeks after the end of CRS, I exposed the rats to an acute stress and investigate whether the animal acute responsiveness could be altered by the previous stress exposure. I focused my molecular analyses on modulators of the oxidative balance, recognized to have a role in the etiology of mental diseases, and on the protective therapeutic role of the antipsychotic lurasidone.

3. Materials and Methods

3.1 Experimental paradigms

3.1.1 Experiment 1: Prenatal stress and EAE induction

Animals

Adult female C57BL/6 pregnant mice at gestational day (GD) 14 were purchased from a commercial breeder (Charles River, France). Upon arrival, the animals were singly housed with food and water freely available and were maintained on a 12-h light/dark cycle in a constant temperature (22 ± 2^{o}C) and humidity (50 ± 5%) conditions. All animal experiments were conducted according to the authorization from the Health Ministry n.1136/2016PR in full accordance with the Italian legislation on animal experimentation (DL 26/2014) and adherent to EU recommendation (EEC Council Directive 2010/63). All efforts were made to minimize animal suffering and to reduce the number of animals used.

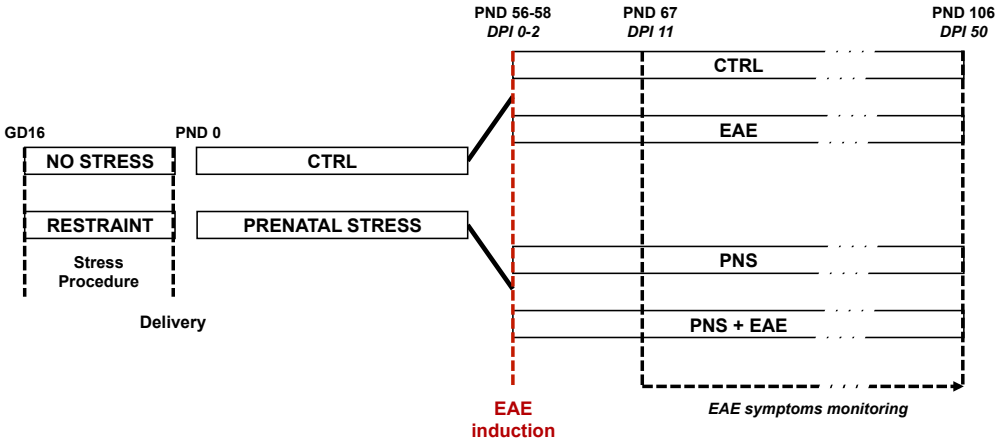
Experimental conditions and stress procedure

Ten pregnant dams were randomly selected for exposure to restraint stress, from GD16 until delivery. Briefly, the animals were subjected to two (GD16 and GD17) or three (GD18 and GD19) daily stress sessions at 10.00 a.m. and 14.00 p.m. (GD16 and GD17) or 9.30 a.m., 12.30 p.m. and 15.30 p.m. (GD18 and GD19), during which they were placed in plastic cylinders (12cm long and 4cm diameter) for 45 minutes under bright light (3000 lux). The control pregnant females were left undisturbed in their home cages in the same room where the stress was performed. At weaning (post-natal day 21, PND21) female pups were selected from control and stressed mothers and subjected to one of four

experimental conditions: (i) mice born from non-stressed dams-NOPNS/CTRL (n=12), (ii) mice born from non-stressed dams exposed to the EAE model-NO PNS/EAE (n=15), (iii) mice born from stressed dams-PNS/CTRL (n=15), (iv) mice born from stressed dams exposed to EAE-PNS/EAE (n=16). All animals were socially housed (n=4 per cage) under standard laboratory conditions.

Experimental Autoimmune Encephanomyelitis (EAE)

EAE was induced in 8-week-old female by subcutaneous immunization in the flanks and in the tail base with 300 µg of myelin oligodendrocyte glycoprotein (MOG35-55, Espikem) per mouse in IFA (Sigma Aldrich) supplemented with 8 mg/ml of Mycobacterium tuberculosis (strain H37Ra, Difco). Mice immunized received 500 ng of pertussis toxin (PTX, Duotech) intravenously the day of the immunization and 48 h later. Animals were daily weighted and scored for clinical symptoms of EAE according the following scale: 0 = healthy, 1 = flaccid tail, 2 = ataxia and/or paresis of hindlimbs, 3 = paralysis of hindlimbs and/or paresis of forelimbs, 4= tetraparalysis, 5 =moribund or death. Non-EAE controls received PTX injections, as well as the initial injections of emulsion but without the encephalitogen, to ensure that observed effects are due to EAE and not to reactions to the ancillary components used to facilitate disease induction. At PND106/ day post injection 50, all the animals were sacrificed and the spinal cord was dissected for molecular analyses.



Schematic representation of the experimental paradigm

3.1.2 Experiment 2: Prenatal stress and acute stress

Animals

Adult female C57BL/6 pregnant mice at gestational day (GD) 14 were purchased from a commercial breeder (Charles River, France). Upon arrival, the animals were singly housed with food and water freely available and were maintained on a 12-h light/dark cycle in a constant temperature (22 ± 2^{o}C) and humidity (50 ± 5%) conditions. All animal experiments were conducted according to the authorization from the Health Ministry n.1136/2016PR in full accordance with the Italian legislation on animal experimentation (DL 26/2014) and adherent to EU recommendation (EEC Council Directive 2010/63). All efforts were made to minimize animal suffering and to reduce the number of animals used.

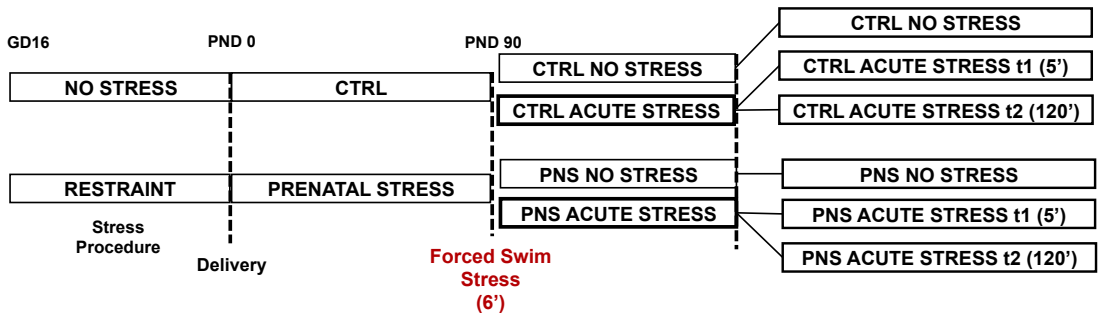
Experimental conditions and stress procedure

Ten pregnant dams were randomly selected for exposure to restraint stress, from GD16 until delivery. Briefly, the animals were subjected to two (GD16 and GD17) or three (GD18 and GD19) daily stress sessions at 10.00 a.m. and 14.00 p.m. (GD16 and GD17) or 9.30 a.m., 12.30

p.m. and 15.30 p.m. (GD18 and GD19), during which they were placed in plastic cylinders (12cm long and 4cm diameter) for 45 minutes under bright light (3000 lux). The control pregnant females were left undisturbed in their home cages in the same room where the stress was performed.

At weaning (post-natal day 21, PND21) male pups were selected from control and stressed mothers and to one of four experimental conditions: i) mice born from non-stressed dams not exposed to acute stress-NO PNS/NO AS, (ii) mice born from non stressed mice exposed to acute stress and sacrificed after 5 minutes-NOPNS/AS 5', (iii) mice born from non stressed mice exposed to acute stress and sacrificed after 2 hours-NOPNS/AS 2h, (iv) mice born from stressed dams not exposed to acute stress-PNS/NO AS, (v) mice born from stressed mice exposed to acute stress and sacrificed after 5 minutes-PNS/AS 5', (vi) mice born from stressed mice exposed to acute stress and sacrificed after 2 hours-PNS/AS 2h. All animals were socially housed (n=4 per cage) under standard laboratory conditions.

AS groups, once adult (PND90) were exposed to an acute stress, the Forced Swim Stress, for 6 minutes, during which the mice were placed in a tank with a capacity of 2L, filled with water in a way that the animals were not able to touch the bottom of the tank, either with their feet or tail or jump out of it.



Schematic representation of the experimental paradigm

3.1.3 Experiment 3: Social defeat stress and 2rTBI

Animals

Adolescent male C57BL/6J mice at 3 weeks of age were purchased from the Jackson Laboratory. Mice were given one week of acclimation, individually housed with a reversed 12-hour light – 12-hour dark cycle and provided food and water ad libitum.

Experimental conditions and stress procedure

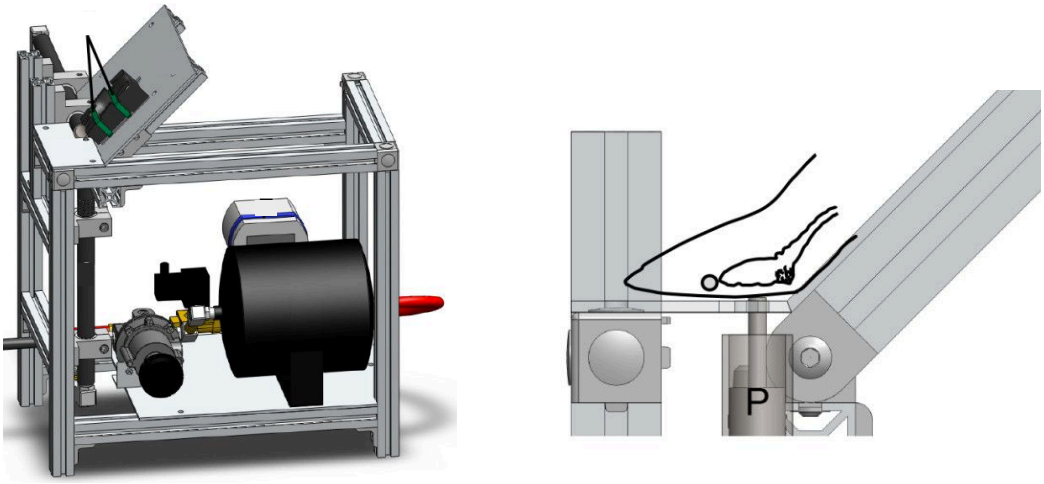
At 4 weeks, mice were randomly assigned to one of the following group: (i) mice not exposed to the social defeat paradigm without injury-No Stress/sham (n=10), (ii) mice not exposed to the social defeat paradigm that received the repetitive concussion-No Stress/rTBI (n=10), (iii) mice stressed with the social defeat paradigm without injury-Stress/sham and (n=12) (iv) mice stressed with the social defeat paradigm that received the repetitive concussion-Stress/rTBI (n=12).

Mice from group iii and iv were exposed to the social defeat stress for 10 consecutive days. In each daily session the test mice was exposed an older, aggressive, dominant sex-matched mouse (CD1, Charles River) for 10 minutes. In details, the experimental mouse was placed in the home cage of the dominant mouse. After the 10 minutes stress, mice were left in the same cage with the aggressor but separated by a perforated plexiglass divider to allow only sensory contact for 24 hours. Control (non-stressed) animals were pair housed in cages with one mouse per side of the perforated divider. All control mice were rotated daily, similarly to mice undergoing defeat, but without physical contact with their cage mates.

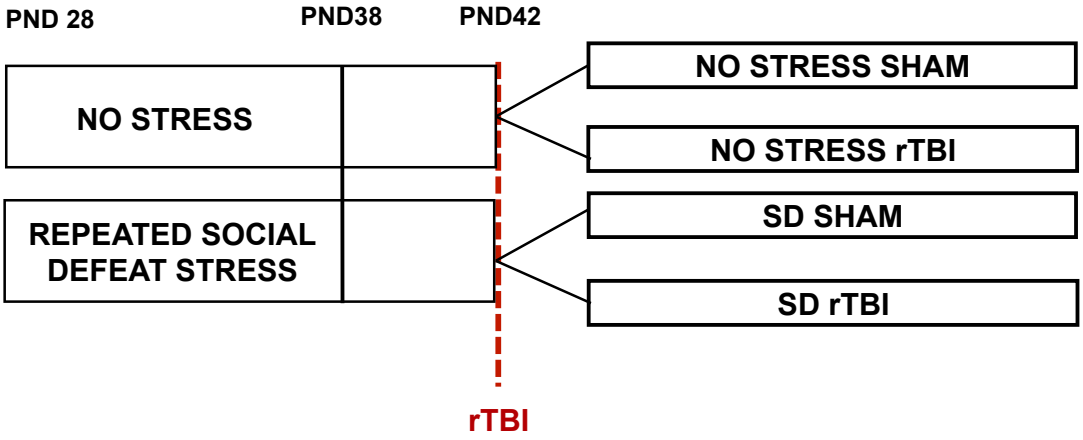
All animal experiments were conducted in compliance with animal protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco (UCSF), following the National Institutes of Health Guidelines for animal care.

Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) repetitive brain injury

Experimental repetitive mild traumatic brain injury (rTBI) was induced in 6 weeks old mice using the Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) TBI model. The animals were anesthetized using isoflurane (2%) in oxygen 1L/minute during the procedure. rTBI animals were subjected to 2 mild closed head injuries using the CHIMERA device as previously reported (Namjoshi et al., 2017). Briefly, rTBI animals were placed supinely into an angled holding platform without any shaving of the head or incision into the skin so that their head was level with the piston target hole while aligning the eyes, ears and nose such that the impact was centered on the dorsal convexities of the skull, targeting a 5 mm area surrounding bregma. A nose cone delivering isoflurane was removed just prior to the impact. Impact was initiated using Real Term software that was connected to a system including air tank, pressure regulator, digital pressure gauge, two-way solenoid valve, and piston. The impact was administered using an air pressure of 2.95 PSI, resulting in an impact energy of 0.5 J from the 5 mm, 50 g piston. Animals were moved to an incubator immediately after the impact and monitored until fully recovered. rTBI animals received an injury once per day for 2 days with a 24 hour interval in between impacts. Sham mice were exposed to the same isoflurane anesthesia paradigm (~ 8 minutes of anesthesia in total) without sustaining an impact. All animals regained the righting reflex in 5 minutes or less. Skull fractures, seizures, apnea or mortality were not observed in any animals, and no animals were excluded from the study due to injury parameters.



Schematic representation of the CHIMERA apparatus



Schematic representation of the experimental paradigm

3.1.4 Experiment 4: Chronic restraint stress and acute stress

Animals

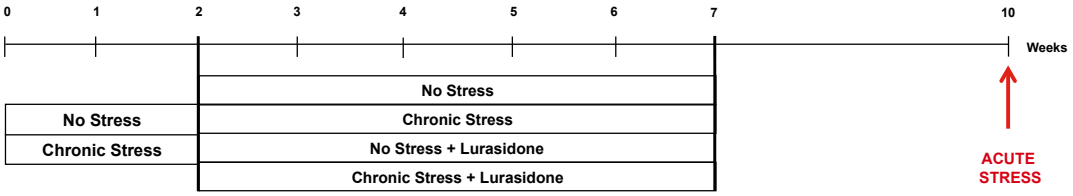
Adult male Sprague-Dawley rats (Charles River, Italy) were brought into the laboratory two weeks before the start of the experiment. Rats were housed with food and water freely available and were maintained on a 12-h light/dark cycle, socially housed (n=3/4 per cage) in a constant temperature (22 ± 2^{o}C) and humidity (50 ± 5%) conditions. All procedures used in this study have conformed to the rules and principles of the 2010/63/UE Directive, according to the authorizations from the Health Ministry n 151/2017-PR.

Experimental conditions and stress procedure

After two weeks of adaptation to laboratory and housing conditions, rats were randomly selected for exposure to chronic restraint stress (CRS) and divided in the following experimental groups: i) naive animals, treated with saline and not exposed to acute stress-No Stress/VEH/NO AS (n=10); ii) naïve animals, treated with saline exposed to acute stress-No stress/VEH/AS (n=10); iii) naïve animals, chronically treated with lurasidone not exposed to acute stress-No Stress/LUR/NO AS (n=10); iv) naïve animals, treated with lurasidone and exposed to acute stress-No Stress/LUR/AS (n=10); v) stressed animals, treated with saline not exposed to acute stress-CRS/VEH/NO AS (n=10); vi) stressed animals, treated with saline exposed to acute stress-CRS/VEH/AS (n=10); vii) stressed animals, chronically treated with lurasidone not exposed to acute stress-CRS/LUR/NO AS (n=10); viii) stressed animals, treated with lurasidone and exposed to acute stress-CRS/LUR/AS (n=10).

CRS rats were exposed to an unpredictable chronic restraint stress for 4 weeks. Rats were placed in plastic cylinders for 1 hour two times/day at random hours, to avoid habituation. The dimensions of the restrainer

were similar to the size of the animal, which made the animal almost not able to move. Control rats were left undisturbed in their home cages in the same room where the stress was performed. At the end of the stress procedure, all the animals were left undisturbed for three weeks of recovery (washout). Following the washout period, rats were exposed to one hour of acute restraint stress (AS).



Schematic representation of the experimental paradigm

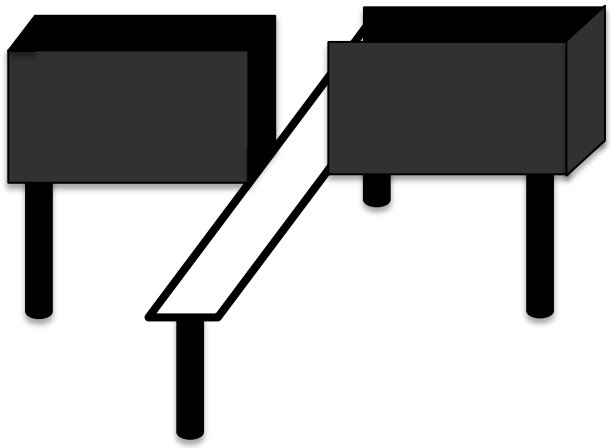
3.2 Behavioural tests

Nest building assessment (Experiment 1)

The nest building assessment test was performed to investigate changes in the well-being of the animals. Raw material (nesting paper) was provided to all the pregnant females and, the day after the birth, the complexity of the nest has been scored by four different blind operators according to the following scale: 0 = the animal has not manipulated the building material; 1 = the material has been manipulated but the position of the nest is not clear (sparse paper on the bottom of the cage); 2 = flat nest, without vertical walls; 3 = "cup" nest with walls less than half the height of a nest with a full dome; 4 = nest with incomplete dome; 5 = nest with full dome.

Elevated Plus Maze (Experiment 3)

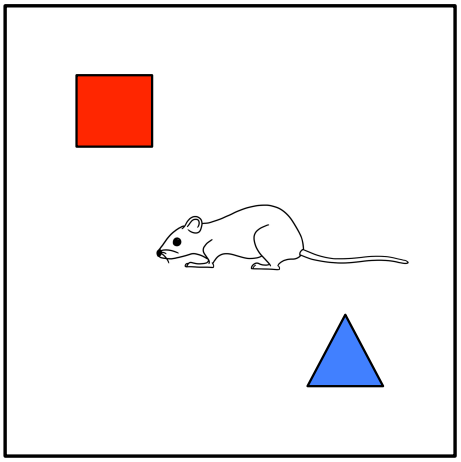
Animals were tested 7 days post injury using an elevated plus-shaped maze, raised 40 cm above the floor in a room with bright lighting. The surface of the maze consisted of two closed arms opposite to each other (30.5 cm in length) enclosed in black plastic. The other two perpendicular arms were open (35 cm in length) with white lights illuminating the open arms. Mice were placed in the center (4.5 cm square) of the maze and allowed to explore for 5 minutes while their location and activity were recorded. The maze was cleaned with 70% ethanol between animals. The time in the open area (which included time both in the open arms and center which is uncovered) was recorded and scored using a video tracking and analysis setup (Ethovision XT 8.5, Noldus Information Technology). Mice that fell off the maze during testing were excluded from analysis.



Schematic representation of Elevated Plus Maze apparatus

Novel Object Recognition (Experiment 3)

Mice were tested for NOR 3 week post injury. An open arena (30 cm × 30 cm×30 cm; L×W×H) was placed in a dimly lit behavior test room with an overhead camera. The mice were allowed to explore the open arena for 10 min for two consecutive days. On day 3, two identical objects were placed in the arena and mice were allowed to explore for 5 min. On day 4, one of the objects was replaced by a novel one and mice were allowed to explore for 5 min. Exploratory behavior was defined as the animal directing its nose toward an object at a distance less than 2 cm. Objects were secured in the arena with magnets. Arena and objects were wiped with 70 % ethanol between trials to eliminate odor cues. Trials were recorded by the overhead camera and analyzed by an automatic video tracking system (EthoVision, Noldus) for movement tracking and by manual scoring for exploratory behavior. Discrimination index was calculated using the formula: (time exploring novel object − time exploring familiar object)/total exploring time.



Schematic representation of Novel Object Recognition test

3.3 Molecular Analyses

Molecular analyses were conducted in the cervical portion of the spinal cord (Exp 1) in the hippocampus (Exp 2, Exp 3 and Exp 4) of the experimental animals.

3.3.1 RNA preparation and gene expression analyses

For gene expression analyses, total RNA was isolated from CNS samples by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l.; Segrate, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for real-time polymerase chain reaction (PCR) as previously reported (Rossetti et al., 2018). Briefly, an aliquot of each sample was treated with DNAse to avoid DNA contamination and subsequently analyzed by TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l.) using the iScript one-step RT-PCR kit for probes (Bio-Rad Laboratories S.r.l.). Samples were run in 384-well format in triplicate as multiplexed reactions with a normalizing internal control (β-actin). Thermal cycling was initiated with incubation at 50 °C for 10 min (RNA retrotranscription), and then at 95 °C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 sec to enable the melting process, and then for 30 sec at 60 °C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression versus the control group. Specifically, fold change for each target gene relative to β-actin was determined by the $2^{-\Delta(\Delta CT)}$ method, where ΔCT = CT,target – CT, β -actin and Δ(ΔCT) = CT, exp. group - CT, control group and CT is the threshold cycle. For graphical clarity,

the obtained data were then expressed as percentage versus control group, which has been set at 100%.

Gene	Forward primer	Reverse primer	Probe
β-Actin	CACTTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG	TCTGGGTCATCTTTTCACGGTTGGC
Arc	GGTGGGTGGCTCTGAAGAAT	ACTCCACCCAGTTCTTCACC	GATCCAGAACCACATGAATGGG
cFOS	TCCTTACGGACTCCCCAC	CTCCGTTTCTCTTCCTCTTCAG	TGCTCTACTTTGCCCCTTCTGCC
Tgf-β	GCTGGCAGTAGCTCCCCTATTT	TTGAGGTTGAGGGAGAAAGCAG	GTGGTATACTGAGACACCTTGGTGTC
BDNF iso IV	AGCTGCCTTGATGTTTACTTTG	CGTTTACTTCTTTCATGGGCG	AGGATGGTCATCACTCTTCTCACCTGG
Long BDNF	GTTGTCATTGCTTTACTGGCG	AATTTTCTCCATCCCTACTCCG	AATCTACCCCTCCCATTCCCCGT
BDNF iso VI	GGACCAGAAGCGTGACAAC	ATGCAACCGAAGTATGAAATAACC	ACCAGGTGAGAAGAGTGATGACCATCC
Total BDNF	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG

Gene	Assay ID
Il-1β	Mm00434228_m1
Tnf-α	Mm00443258_m1
Il-6	Mm00446190_m1
Nrf2	Rn00582415_m1
Gpx1	Rn00577994_g1
Gpx4	Rn00820816_g1
Mt-1α	Rn00821759_g1
Srxn1	Rn04337926_g1
Nox2	Rn00675098_m1

Table 1: Sequences of forward and reverse primers and probes used in Real-time PCR analyses and purchased from Eurofins MWG-Operon and probes purchased from Life Technologies, which did not disclose the sequences.

3.3.2 Protein extraction and western blot analyses

CNS samples were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer (containing 0.32 M sucrose, 1 mM MgCl2, 1mM NaHCO3, 10 mM HEPES solution, and 0.1 mM phenylmethylsulfonyfluoride in presence of a complete set of proteases [Roche] and phosphatase [Sigma-Aldrich] inhibitors) and then sonicated for 10 seconds at a maximum power of 10% to 15% (Bandelin Sonoplus). The homogenate was clarified (1000 g; 10 minutes), obtaining a pellet (P1) enriched in nuclear components, which was resuspended in a buffer (20 mM HEPES, 0.1mM dithiothreitol, 0.1 mM EGTA) supplemented with protease and phosphatase inhibitors. The supernatant (S1) was then centrifuged (13000g; 15 minutes) to obtain a clarified fraction of cytosolic proteins (S2). The pellet (P2), corresponding to the crude membrane fraction, was resuspended in the same buffer used for the nuclear fraction. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin (BSA) as calibration standard.

Equal amounts of protein (ranging from 10 to 17 ug) were run under reducing conditions on polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes. Unspecific binding sites were blocked with 10% nonfat dry milk; then the membranes were incubated overnight with the primary antibodies and for1 or 2 hours at room temperature with a peroxidase-conjugated anti-rabbit or anti-mouse IgG. Immunocomplexes were visualized by chemiluminescence using the ECL ETA C 2.0 (Cyanagen) or ECL SUN (Cyanagen). Results were standardized using β-actin as the internal control, which was detected by evaluating the band density at 43 kDa. Protein levels were calculated by measuring the optical density of the immunocomplexes using chemiluminescence (Chemidoc MP Imaging System, Bio-Rad Laboratories). To ensure that autoradiographic bands would be in the

linear range of intensity, different exposure times were used.

3.3.3 Immunohistochemistry

Experiment 1

Three animals from each experimental condition were dedicated to immunofluorescence staining. Mice were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) and perfused transcardially with 0,1 M EDTA (Sigma Aldrich) in saline followed by 4% neutral buffered formalin (Sigma Aldrich) in deionized water. Spinal cords were collected and post-fixed for 1 hour in the same solution at 4°C, cryoprotected in 30% sucrose for 24 hours, embedded in OCT and then frozen at -80°C. Spinal cords were cut transversally into 20 µm-thick sections with a cryostat and processed for immunofluorescence. Slides were incubated for 45 minutes at room temperature with a blocking solution composed by 10% normal goat serum and 0.1% triton X-100 in phosphate buffered saline (PBS). Then, the sections were incubated with rabbit polyclonal anti-GPR17 (1:2500, custom antibody produced by PRIMM, Milan, Italy) overnight at 4°C in PBS with 5% goat normal serum and 0.1% Triton X-100. Following primary antibody incubation, the sections were washed and incubated with the biotinylated secondary antibody (Vector Labs, Burlingame, USA) for 1 hr at room temperature. GPR17 labeling was detected with the high sensitivity tyramide signal amplification kit (Perkin Elmer, Milan, Italy) according to the manufacturer's instruction. Hoechst 33528 was used to visualize cell nuclei. After processing, sections were mounted on microscope slides with fluorescent mounting medium (Dako, Milan, Italy).

For each animal 2 sections from the cervical spinal cord were entirely acquired at 10X magnification and reconstructed with Adobe Photoshop CC. In each section GPR17 positive cells were counted in the whole white matter.

Experiment 3

After sacrifice, one brain hemisphere from each experimental mouse was immediately put into 4% PFA at 4° C, fixed overnight and then switched into 30% sucrose solution for at least two days. Hemibrains were then embedded in a 2:1 30% Sucrose/OCT solution, frozen in isopentane and stored at -80°C. Samples were sliced into 20 µm coronal sections with a cryostat and then processed for immunofluorescence. Slices were incubated for 30 minutes at room temperature with a TSA-BB blocking solution and then incubated with rabbit anti-Iba1 (1:400, Wako Pure Chemicals) overnight at 4°C in TSA-BB. Following primary antibody incubation, sections were washed and incubated with AF-568 goat anti-rabbit secondary antibody (1:400, Life Technologies). DAPI was used for nuclear counterstaining. After processing, sections were mounted on microscope slides with ProLong mounting medium (ThermoFisher). Images from hippocampal regionCA1 were taken using a Zeiss Imager Z1 under a 20x objective lens. The area of Iba1+ signal was measured in ImageJ.

Protein	Primary antibody	Secondary antibody
β-ACTIN (43 KDa)	1:10000 1h RT (Sigma)	anti-mouse 1:10000 1h RT
NRF-2 (100KDa)	1:500 O/N 4°C (R&D Systems)	anti-mouse 1:1000 2h RT
IL-10 (24 kDa)	1:1000 O/N 4°C (GeneTex)	anti-rabbit 1:1000 1h RT
PRX-SO3 (22 kDa)	1:2000 O/N 4°C (abcam)	anti-rabbit 1:2000 1h RT
mBDNF (14 kDa)	1:500 O/N 4°C (Icosagen)	anti-mouse 1:1000 1h RT
pAKT (60 kDa) Ser473	1:1000 O/N 4°C (Cell signaling)	anti-rabbit 1:2000 1h RT
AKT (60 kDa)	1:1000 O/N 4°C (Cell signaling)	anti-rabbit 1:1000 1h RT
pmTOR (260 kDa) Ser2448	1:1000 O/N 4°C (Cell signaling)	anti-rabbit 1:1000 1h RT
mTOR (260 kDa)	1:1000 O/N 4°C (Cell signaling)	anti-rabbit 1:1000 2h RT
MAG (100 kDa)	1:1000 O/N 4°C (Cell signaling)	anti-rabbit 1:1000 1h RT
GPR17	1:2500 O/N 4°C (PRIMM)	anti-rabbit 1:1000 2h RT
Iba-1	1:400 O/N 4°C (Wako Pure Chemicals)	anti-rabbit 1:400 2h RT

Table 2: Conditions of the antibodies used in western blot analyses and immunohistochemistry analyses (O/N: over/night; RT: room temperature).

3.4 Statistical Analyses

3.4.1 Behavioral Results

Experiment 1

Analyses of body-weight gain -of the mothers and of the pups before and after the EAE induction- and EAE clinical score were performed with Two-way analysis of variance (ANOVA) with repeated measures. Comparisons between stressed animals or non stressed mice in nest building assessment test score, number of pups, ratio between female and male newborn and day of the EAE onset were analyzed by two-tailed Unpaired t test.

Experiment 2

Analysis of body-weight gain was performed with Two-way analysis of variance (ANOVA) with repeated measures.

Experiment 3

Analyses of time spent in the open arms and center (EPM) and of the discrimination index (NOR) were performed with Two-way analysis of variance (ANOVA) followed -when appropriate- by a Single Contrast Post Hoc Test (PLSD). Significance for all was assumed for $P<0.05$. Data are presented as means ± SEM.

3.4.2 Molecular Results

For exp 1, 2 and 3, the paradigm effects on the mRNA and protein levels or the signal density of the molecular targets were evaluated with a Two-way ANOVA followed -when appropriate- by a Single Contrast Post Hoc Test (PLSD).

In exp 1, differences in the GPR17 positive cell count and MAG protein levels were evaluated using two-tailed Unpaired t test.

In exp 4, the three-way ANOVA with PLSD was used to investigate the

effect of chronic restraint stress (NoStress/CRS), of the pharmacological treatment (VEH/LUR) and of the acute restraint challenge (No Stress/AS), as independent factors.

Significance for all was assumed for $P<0.05$. Data are presented as means ± SEM.

4. Results

4.1 Prenatal stress reshapes spinal myelination affecting BDNF signaling in the experimental autoimmune encephalomyelitis model of multiple sclerosis.

Maria Serena Paladini, Davide Marangon, Andrea C. Rossetti, Alice Guidi, Giusy T. Coppolino, Camilla Negri, Maria Pia Abbracchio, Davide Lecca and Raffaella Molteni

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4.1.1 Introduction

Stress experience has been consistently established to be a major environmental factor in the etiology of several neurological and psychiatric diseases (Gradus, 2017). Thus, living stressful situations can lead to several molecular alterations that can eventually evolve from a normal adaptive body reaction to a medical condition (Davis et al., 2017). This different trajectory may depend on several variables such as the genetic background of the subject, the socio-economic context, the nature, severity and duration of the stressful experience and the time when the stress occurs. In particular, the gestational period is a so-called “window of vulnerability” (Briscoe et al., 2016) and the exposure to adverse events during pregnancy has been shown to impact not only on maternal health but also to have a deep long-lasting influence on the offspring neurodevelopment, leading to an enhanced susceptibility to diseases and dysfunctions during adulthood (Zucchi et al., 2013; Coe and Lubach, 2005; Entringer et al., 2015). Underlying this effect named “fetal programming” (Barker, 1998; Kwon and Kim, 2017) it has been hypothesized that prenatal stress (PNS) can leave a signature in the progeny by affecting neural plasticity. In line with this hypothesis, prenatal stress exposure has been associated to alterations of the neurotrophin Brain-derived neurotrophic factor (BDNF), a crucial player in neurodevelopment and neuronal plasticity known to be involved in several neurodegenerative and psychiatric diseases (Autry and Monteggia, 2012; Zuccato and Cattaneo, 2009). For example, PNS has been found to reduce BDNF gene expression in the amygdala and hippocampus of rats at weaning and during adulthood (Boersma et al., 2014), to increase BDNF (exon IV) DNA methylation in the medial prefrontal cortex of adult male rats (Blaze et al., 2017), and to decrease the neurotrophin protein levels in the hippocampus of both female and male rats (Yeh et al., 2012). Of note, changes in BDNF expression were

also found in the spinal cord of prenatally stressed adult rats (Winston et al., 2014). Even though the more robust evidences for BDNF modulation by early life stress derive from preclinical studies, it has been also reported in humans that maternal experiences of chronic stress -such as war trauma- are associated with alterations of BDNF methylation in both newborn and maternal tissues (Kertes et al., 2017) and the level of the neurotrophin in the amniotic fluid during pregnancy is positively correlated to maternal early adversity exposure (Deuschle et al., 2018).

On these bases, the aim of our study was to investigate the potential long-lasting impact of PNS exposure on the susceptibility to pathologies known to be characterized by alterations of neural function and plasticity, such as multiple sclerosis (MS), an autoimmune disease whose incidence is greatly increasing among young individuals, starting from adolescents (GBD 2016 Multiple Sclerosis collaborators). The contribution of environmental factors such as stress in prompting MS or influencing its manifestations and course is not clearly elucidated (Heesen et al., 2007). Specifically, little is known about the mechanisms by which adverse events during -or around- the gestation period may increase the susceptibility to MS in the progeny. Indeed, to the best of our knowledge, only few clinical studies have linked MS risk with features of maternal behavior including breastfeeding duration (Ragnedda et al., 2015; Conradi et al., 2013), delivery mode (Maghzi et al., 2012; Nielsen et al., 2013) or vitamin D intake (Mirzaei et al., 2011). In this regard only one report indicating a possible -but not statistically significant- association between stressors, such as late prenatal maternal care and maternal illness during pregnancy, and MS has been published so far (Gardener et al., 2009). At preclinical level, most studies using the experimental autoimmune encephalomyelitis (EAE) mouse -the most commonly model for MS- are focused on the long-term effects of neonatal manipulations (Krementsov and Teuscher, 2013;

Teunis et al., 2002; Columba-Cabezas et al., 2009, Case et al., 2010) but the influence of stress during gestation on EAE has not yet been investigated.

With these premises, the aim of this study has been to evaluate the impact of a prenatal stress exposure on EAE course at adulthood. Specifically, given that MS affects women twice as often as men (Harbo et al., 2013), we induced EAE in the female adult progeny of dams exposed to a stressful manipulation during the last days of gestation and we scored the clinical symptoms in comparison with un-stressed cohorts. Moreover, in order to clarify the molecular mechanisms underlying the stress effect, specific molecular analyses have been performed in the spinal cord. In particular, given the demyelinating nature of the encephalomyelitis, we first focused our analyses on markers of different stages of olygodendrocyte maturation and thereafter on the upstream Akt/mTOR signaling pathway, relevant for myelination itself, pinpointing the neurotrophin BDNF as a potential player in the long-lasting influence of PNS on EAE development and MS vulnerability.

4.1.2 Results

4.1.2.1 Behavioral Results

4.1.2.1.1 Impact of stress exposure on dams and female offspring

To establish whether restraint stress exposure affects proper gestation course, starting from GD17 until the 38th day post-partum, we monitored the body weight of control and stressed pregnant mice as well as their capability to build a nest, using the nest test as an indicator of animal wellbeing. As shown in figure 1, no difference in the body-weight profile (Fig.1A) or in the nest complexity (Fig.1B) was found between stressed and control dams. Likewise, we did not observe significant changes in the number of pups per litter or in the sex ratio (Fig.1C, D).

Subsequently, to establish the lasting effects of prenatal stress (PNS) also in the offspring after weaning, we monitored the weight of female pups from PND 24 to PND 56, finding that early stress reduced the body weight of female pups born from stressed dams compared to their non-stressed littermates ($F_{1,56}$= 25.78, P<0.001; Fig.1E).

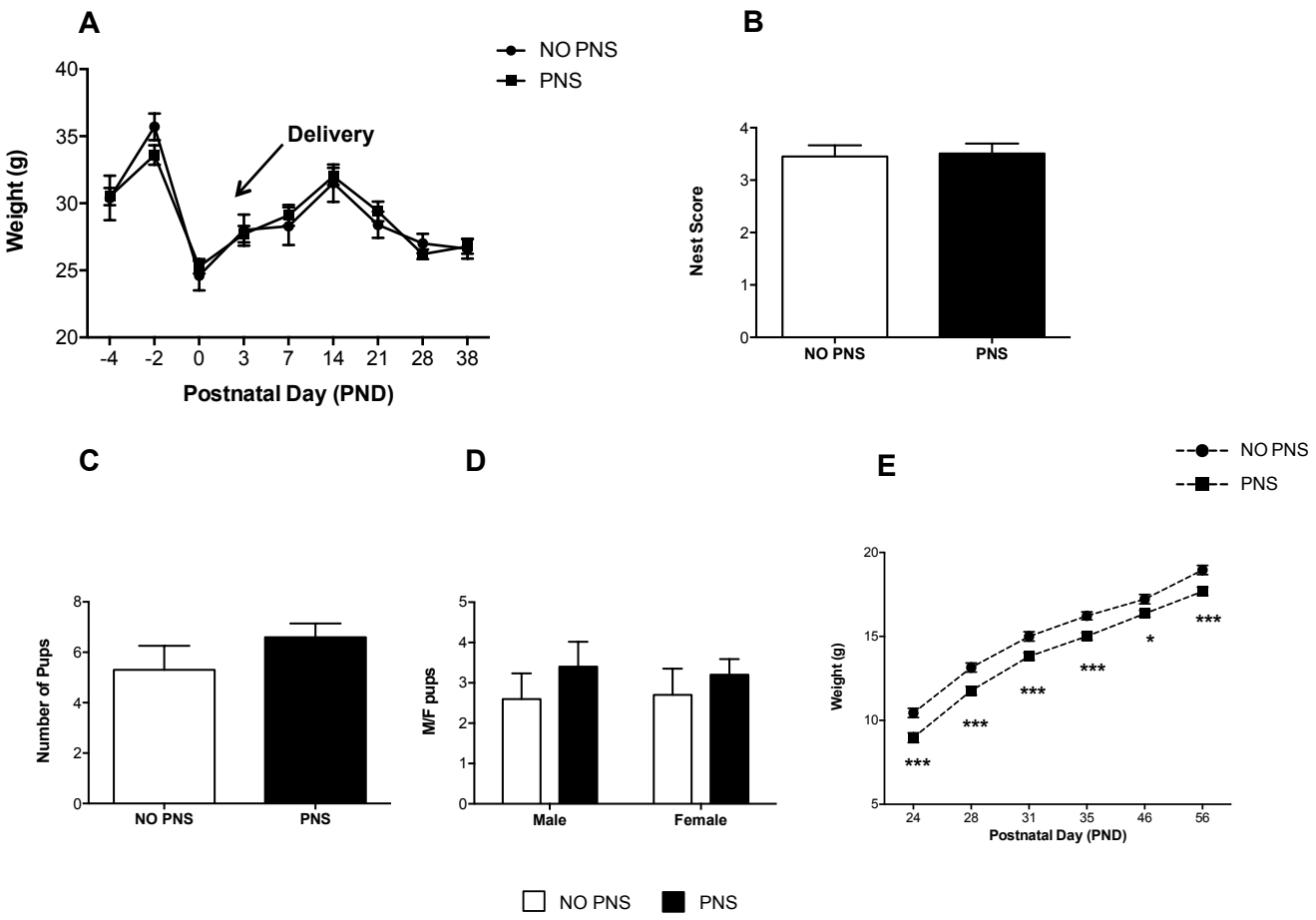

Fig.1 Impact of stress exposure on dams and female offspring

(A) Body weight of pregnant control (NO PNS) and stressed (PNS) dams measured during gestational stress and up to 38 days after delivery. (B) Score of the complexity of the nests built by control and stressed mothers and assessed the day after delivery. (C) Total number of pups and (D) ratio between male and female pups born from control and stressed mothers. (E) Body weight of female pups born from control and stressed mothers monitored from PND 24 to PND 56.

For all the analyses the data are expressed as mean of the examined variable ± SEM of independent determinations. *P<0.05, ***P<0.001 vs. NO PNS (Two-way ANOVA with Fisher's LSD).

4.1.2.1.2 Long-term effect of prenatal stress exposure on EAE clinical signs and course

To assess whether PNS could affect EAE course and severity, EAE female pups were daily weighted and scored using the 0-5 grading system for clinical assessment from day post-immunization (DPI) 1 to DPI 50. The clinical profile of EAE symptoms is reported in figure 2, where the body weight changes (Fig.2A) and disability score (Fig.2C) during the four phases of EAE progression -onset, acute phase, recovery and chronic phase- are shown. The statistical analysis indicated that pups born from stressed dams weighted less than their littermates in the acute phase of the clinical signs development ($F_{1,26}$ = 4.979, P<0.05). It is noteworthy that PNS reduced also the body weight of non-immunized control mice throughout adulthood ($F_{1,25}$= 15.05 P<0.001; Fig.2B). Furthermore, clinical manifestations of EAE in prenatally stressed mice were enhanced as compared to the control EAE group as displayed by the increased EAE score over time, especially during the acute phase ($F_{1,22}$ = 4.630, P<0.05) and the following recovery phase ($F_{1,22}$ = 7.667, P<0.05). The cumulative disease score (CDS) was also higher in the PNS/EAE group (P=0.0662; Fig.2D). No difference was observed in the day of onset and in the maximum score (CS max) (Fig.2E, F). Taken together, these behavioral results provide clear evidences that PNS enhances the susceptibility to develop a more severe EAE in the female offspring.

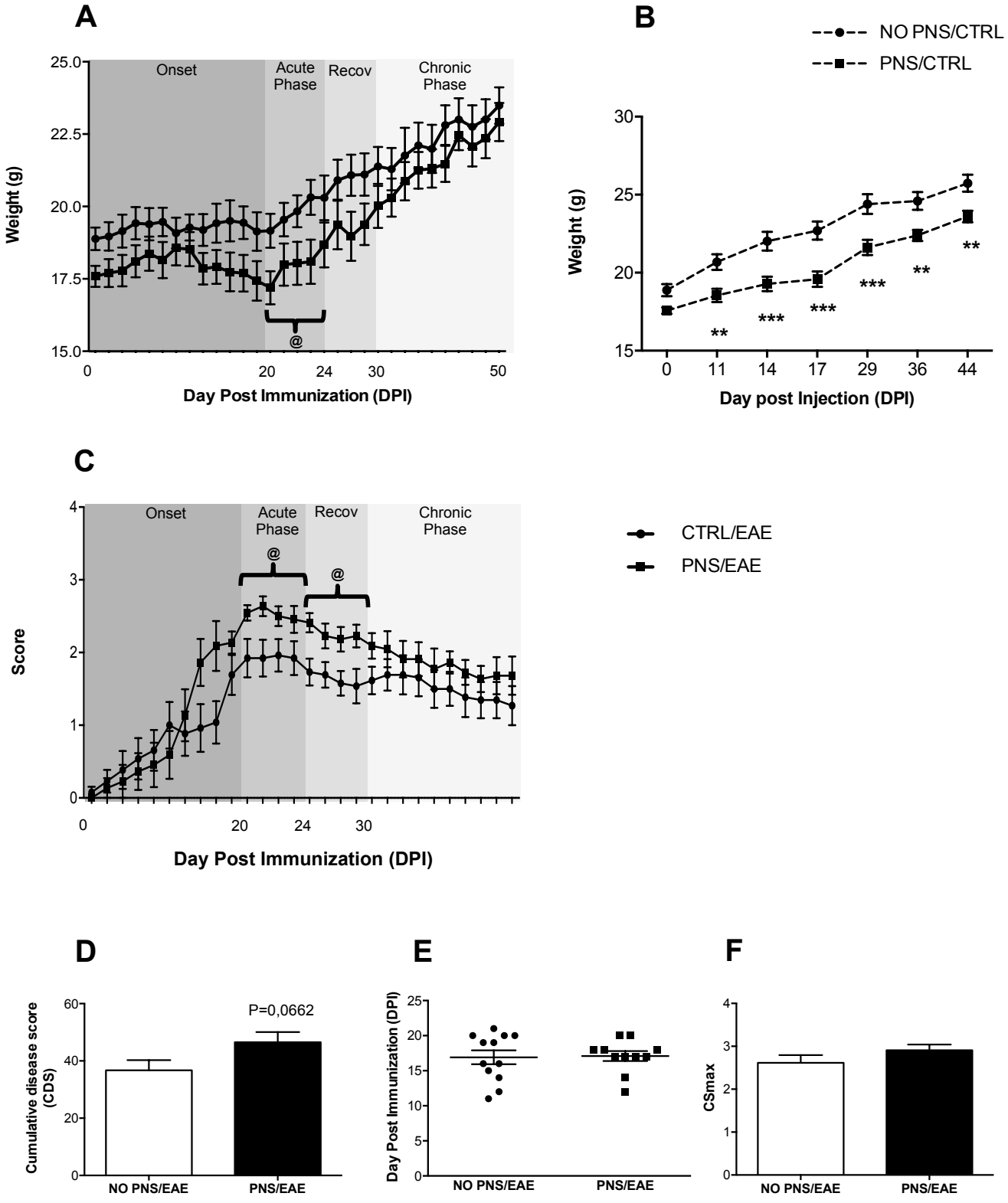
A
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Fig.2 Long-term effect of prenatal stress exposure on EAE clinical signs and course

(A) Body weight and (C) clinical score of EAE animals monitored from DPI 0 to DPI 50 throughout the 4 phases of EAE course (onset, acute phase, recovery, chronic phase). (B) Body weight of non-immunized control and stressed animals from DPI 0 to DPI 44. (D) Cumulative disease score CDS, (E) day of EAE symptoms onset and (F) maximum score, CS max in prenatally stressed and non-stressed EAE mice. For all the analyses the data are expressed as mean of the examined variable ± SEM of independent determinations. *P<0.05, **P<0.01, ***P<0.001 vs. NO PNS/CTRL; $^{@}$P<0.05 vs. NO PNS/EAE (Two-way ANOVA with Fisher's LSD).

4.1.2.2 Molecular Results

4.1.2.2.1 *Analyses of GPR17 and MAG in the Spinal Cord*

To gain insight into potential molecular alterations underlying the exacerbated EAE symptomatology observed in mice prenatally exposed to stress, we first analyzed two markers of different stages of oligodendrocytes maturation in the cervical spinal cord: GPR17, a receptor expressed by early oligodendrocyte precursor cells (OPCs) and a key modulator of OPC maturation and myelination (Fumagalli et al., 2015) and MAG, a myelin-associated glycoprotein expressed by mature and differentiated oligodendrocytes.

The count of GPR17 positive cells (GPR17+) in the whole white matter of the cervical spinal cord highlighted a greater number of OPCs in EAE animals prenatally exposed to stress compared to non-stressed EAE mice (+30% vs. NO PNS/EAE, $P<0.01$), as summarized in figure 3, showing reconstructed spinal cord sections of NO PNS/EAE and PNS/EAE mice (Fig.3A) and the numbers of GPR17 positive cells (Fig.3B). Consistently, immunoblot results showed a strong decrease of MAG only in stressed EAE animals ($P<0.01$; -51% vs. NO PNS/EAE, $P<0.05$; Fig.3C), suggesting altered remyelination in the chronic phase of EAE progression and a higher reaction of OPCs that do proliferate, but fail in reaching terminal maturation.

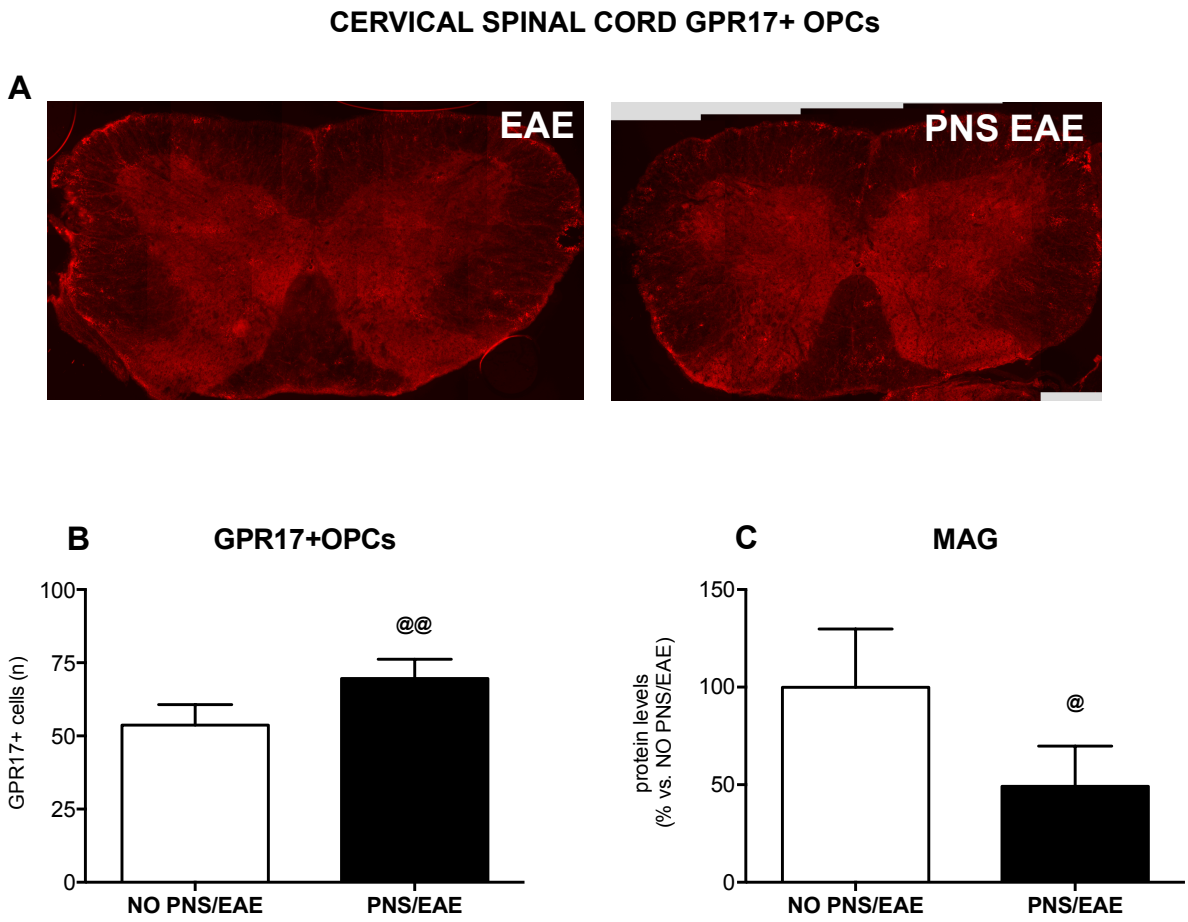

Fig.3 Analyses of GPR17 and MAG in the spinal cord

(A) Reconstructed spinal cord sections of NO PNS/EAE and PNS/EAE mice. GPR17 positive cells (GPR17+) were counted in the whole white matter of the cervical spinal cord (B) whereas MAG protein levels were investigate using western blot analysis (C). GPR17+ counting data are expressed as mean of the examined variable ± SEM of independent determinations. MAG protein levels are expressed as a percentage of non-stressed EAE mice (NO PNS/EAE, set at 100%) and represent the mean ± SEM of independent determinations. $^{@}P<0.05$, $^{@@}P<0.01$ vs. NO PNS/EAE (Unpaired t test).

4.1.2.2.2 Analysis of BDNF/Akt/mTOR signaling

Among the several players involved in the complex process of OPCs differentiation into mature oligodendrocytes, the Akt/mTOR pathway, which can be activated by growth factors, is known to promote differentiation and myelination (Tyler et al., 2009). Indeed, the mammalian target of rapamycin (mTOR), the major downstream player of this pathway, has been implicated in oligodendrocyte differentiation, myelin protein expression, and myelination (Guardiola-Diazet al., 2012) and is an upstream regulator of GPR17 (Tyler et al., 2011; Fumagalli et al., 2015). To elucidate the role of this pathway in the exacerbated EAE outcome in PNS mice, western blot analyses were performed in the spinal cord using antibodies against total and phosphorylated forms of mTOR and Akt.

As shown in figure 4, we observed a significant decrease of Ser2448-phosphorylated-mTOR only in EAE mice exposed to PNS (-58% vs. NO PNS/CTRL, $P<0.05$; Fig.4A) while the total form of the kinase showed a broad reduction trend in all the experimental groups (Fig.4B), suggesting that intrauterine stress exposure had a negative impact on mTOR activation in EAE animals.

Then, we analyzed the levels of the protein kinase Akt which is known to stimulate mTOR activity (Dibble et al., 2015). Despite a significant decrease due only to PNS exposure (-41% vs. NO PNS/CTRL, $P<0.05$), we did not observe any specific modulation of the Serine 473 phosphorylated form of Akt in EAE animals (Fig.4C). On the contrary, in line with our previous observations, we detected a significant reduction of the total protein only in PNS/EAE animals (-26% vs. NO PNS/CTRL, $P<0.05$; Fig.4D) confirming the implication of Akt/mTOR pathway in the impaired neurological score of these animals.

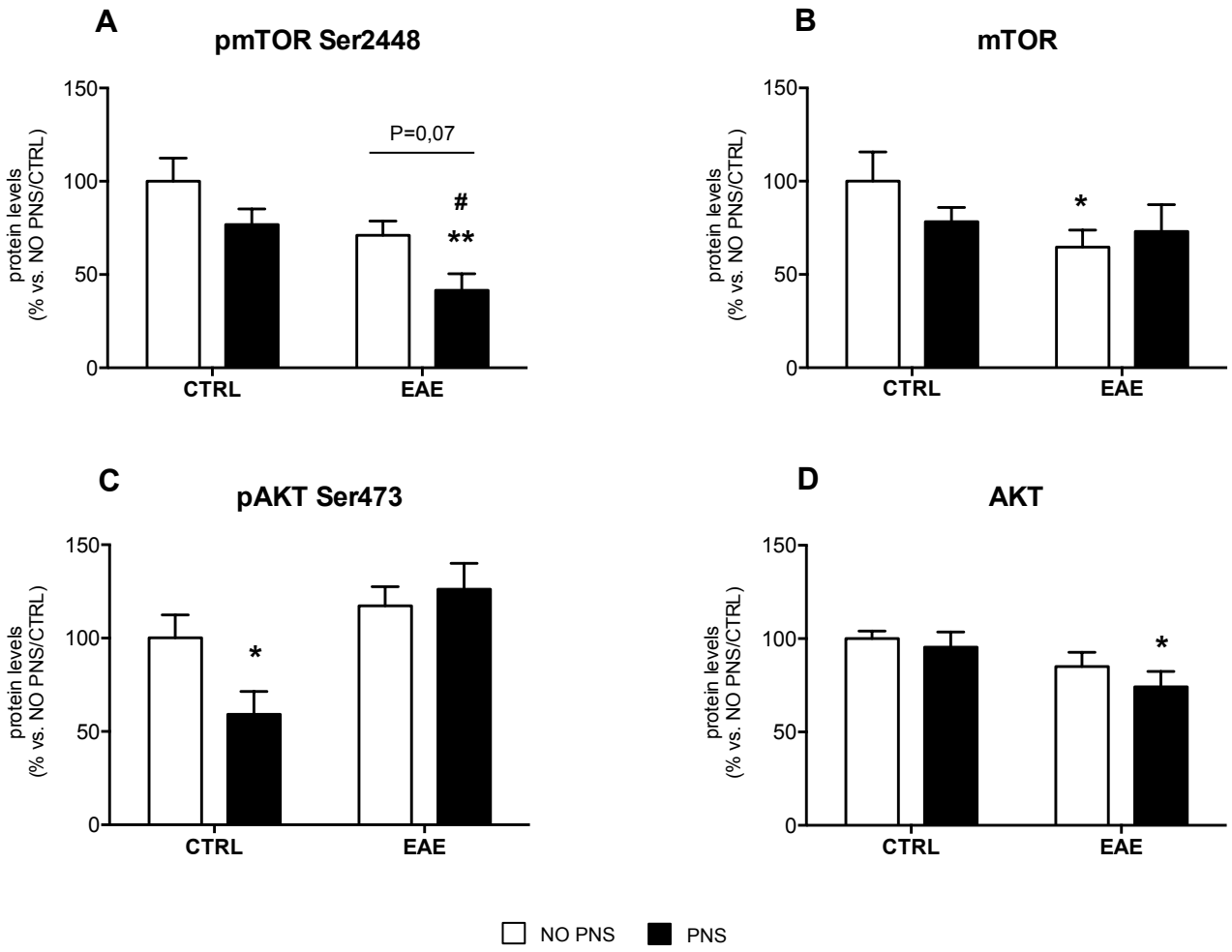

Fig.4 Analysis of BDNF/Akt/mTOR signaling

The protein levels of the kinases phospho-mTOR at Ser2448 (A), mTOR total form (B), phospho-AKT at Ser473 (C) and AKT total form (D) were measured in the cervical spinal cord of CTRL and EAE mice prenatally or not exposed to stress. The data, expressed as a percentage of non-stressed CTRL animals (NO PNS/CTRL, set at 100%), represent the mean ± SEM of independent determinations. *P<0.05, **P<0.01 vs. NO PNS/CTRL; $^{\#}$P<0.05 vs. PNS/CTRL (Two-way ANOVA with Fisher's LSD).

4.1.2.2.3 Analysis of BDNF protein levels

The Akt-mTOR pathway is one of the three main signaling cascades triggered by the binding of the neurotrophin BDNF to its high-affinity tropomyosin receptor kinase B (TrkB), alongside the mitogen-activated protein kinase (MAPK) and the phospholipase Cγ (PLCγ) pathways (Numukawa et al., 2010). Given the role of BDNF in the maintenance of neural plasticity and stress-related diseases, we assessed the protein levels of its mature form in the whole homogenate prepared from the cervical portion of the spinal cord of the mice finding a significant reduction of the neurotrophin in EAE animals. Indeed, as displayed in figure 5, encephalomyelitis decreased BDNF in non stressed EAE animals (-44% vs. NO PNS/CTRL, $P<0.05$), an effect that was even exacerbated in EAE mice prenatally exposed to stress (-58% vs. NO PNS/CTRL, $P<0.01$).

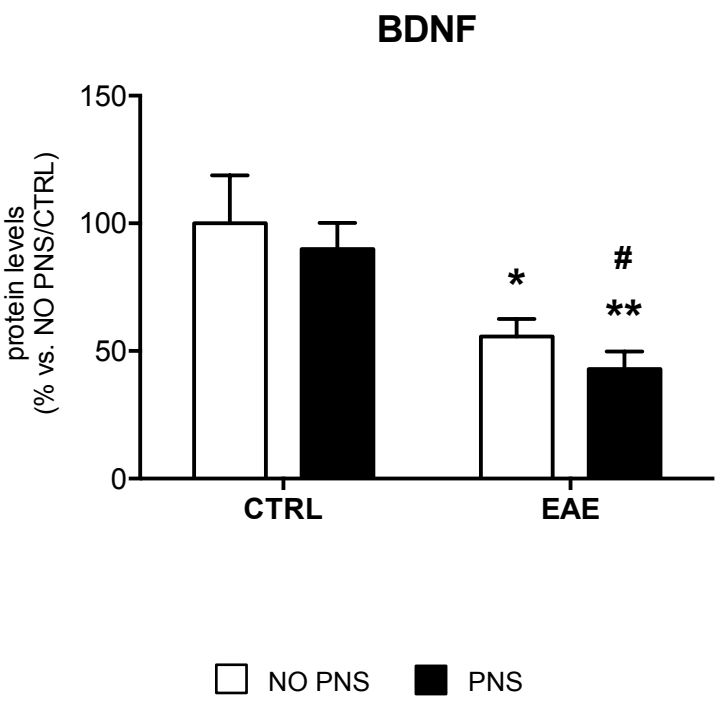

Fig.5 Analysis of BDNF protein levels

The protein levels of the mature form of the neurotrophin BDNF were measured in the cervical spinal cord of CTRL and EAE mice prenatally exposed or not to stress. The data, expressed as a percentage of non-stressed CTRL animals (NO PNS/CTRL, set at 100%), represent the mean ± SEM of independent determinations. *P<0.05, **P<0.01 vs. NO PNS/CTRL; $^{\#}$P<0.05 vs. PNS/CTRL (Two-way ANOVA with Fisher's LSD).

4.1.2.2.4 Analysis of BDNF gene expression

Next, we evaluated if our experimental paradigm could affect BDNF also at transcriptional level. BDNF gene has a complex structure consisting in a number of 5′ untranslated exons alternatively spliced to a common 3' exon that contains the protein coding region. Moreover, within the common 3' exon (exon IX) are located two alternative polyadenylated transcription stop sites that generate two distinct pools of mRNA with either short or long 3′ untranslated regions (3′-UTRs). It

has been proved that the short 3'-UTR mRNAs are restricted to the soma, whereas the long 3'-UTR mRNAs are also localized in dendrites (An et al., 2008). Accordingly, we assessed by Real Time RT-PCR the mRNA levels of total BDNF (transcript IX), the mRNA levels for BDNF long 3'-UTR as well as two major splice variants such as isoforms IV and VI.

We found that the gene expression of total BDNF was significantly modulated in the experimental conditions with a PNS x EAE significant interaction ($F_{1,25}$= 11.43, P<0.01). Indeed, as shown in figure 6, the mRNA levels of total BDNF (Fig.6A) were up-regulated in animals prenatally exposed to stress (+49% vs. NO PNS/CTRL, P<0.001) and reduced by the encephalomyelitis (-26% vs. NO PNS/CTRL, P<0.05) an effect even higher in PNS/EAE animals (-40% vs. NO PNS/CTRL, P<0.01; -60% vs. PNS/CTRL, P<0.001). A partially different profile was observed for the gene expression of the long 3'-UTR pool of transcripts (Fig.6B). Indeed, we found a significant PNS x EAE interaction ($F_{1,22}$ = 12.53, P<0.01) displayed by a strong reduction of long 3'-UTR BDNF only in PNS/EAE mice compared to their control littermates (-41% vs. NO PNS/CTRL, P<0.01), to prenatally stressed mice (-50% vs. PNS/CTRL, P<0.001) and to EAE animals not exposed to stress (-46% vs. NO PNS/EAE, P<0.01). The expression profile of isoform IV was similar to that observed for the total form of the neurotrophin, as indicated in figure 6C by the increase in PNS mice (+35% vs. NO PNS/CTRL, P<0.01) paralleled to a decrease after EAE in both CTRL (-30% vs. NO PNS/CTRL, P<0.05; -48% vs. PNS/CTRL, P<0.001) and prenatally stressed mice (-22% vs. NO PNS/CTRL, P=0.07; -42% vs. PNS/CTRL, P<0.001). Conversely, the modulation of isoform VI was similar to what observed for the long 3' UTR BDNF, with a specific decrease only in the EAE mice prenatally exposed to stress (Fig.6D, -31% vs. NO PNS/CTRL, P<0.05; -32% vs. PNS/CTRL, P<0.05).

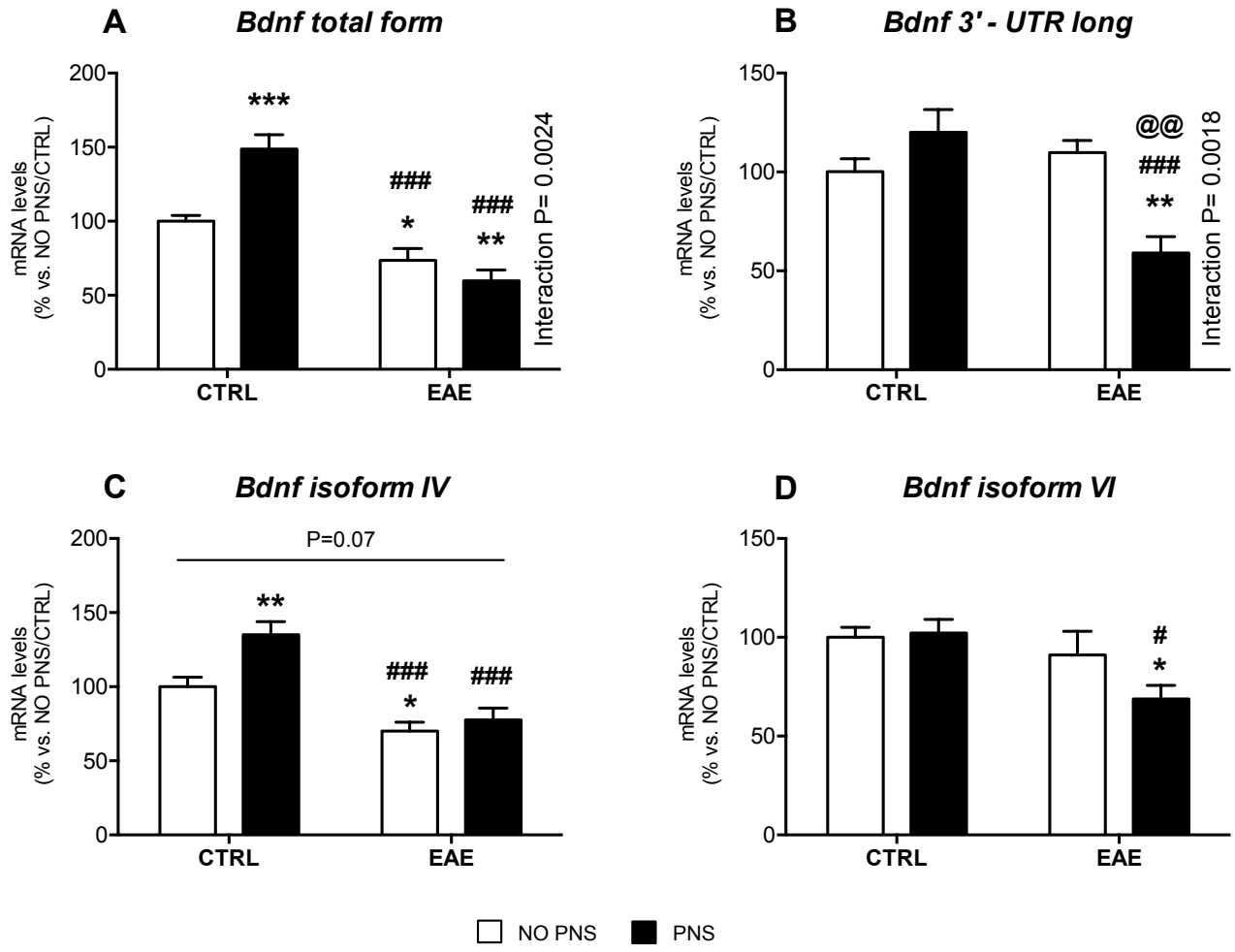

Fig.6 Analysis of BDNF gene expression

The mRNA levels of BDNF total form (A), 3'-UTR long form (B), isoform IV (C) and isoform VI (D) were measured in the cervical portion of the spinal cord of CTRL and EAE mice prenatally exposed to stress or not. The data, expressed as a percentage of non-stressed CTRL animals (NO PNS/CTRL, set at 100%), represent the mean ± SEM of independent determinations.

*P<0.05, **P<0.01, ***P<0.001 vs. NO PNS/CTRL; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. PNS/CTRL; $^{@@}$P<0.01 vs. NO PNS/EAE (Two-way ANOVA with Fisher's LSD).

4.1.3. Discussion

It is well known that adverse events *in utero* can markedly affect neurodevelopment and induce profound long-lasting alterations in the offspring, influencing maturational trajectories and leading to long-lasting alterations eventually resulting in enhanced susceptibility to several diseases later in life (Coe and Lubach, 2005). Indeed, this “fetal programming” hypothesis, by which an insult occurring in a critical period of development has lasting effects (Barker, 1998), has been found to be relevant for metabolic disorders such as obesity and metabolic syndrome (Lauet al., 2011), cardiovascular diseases (Alexander et al., 2015), neuropsychiatric and neurodegenerative disorders (Faaet al., 2016; Modgil et al., 2014). Among this plethora of diseases characterized by susceptibility to fetal programming, little is known about the impact of insults happening to the intrauterine life on multiple sclerosis (MS). While several preclinical studies have been carried out on the effects of environmental risk factors on MS during adulthood (Krementsov and Teuscher, 2013), less is known on the influence of developmental stress exposure and only few studies have addressed this issue by evaluating the impact of earlier post-natal events -such as neonatal handling and cross-fostering- in the experimental autoimmune encephalomyelitis (EAE) model of MS in rats (Leban et al., 1995, Dimitrijević et al., 1994) or in mice (Columba Cabezas et al., 2009; Case et al., 2010). To our knowledge, the only scientific evidence on insults occurring specifically during pregnancy concerns the consequences of maternal infection on EAE course in the offspring (Solati et al., 2012; Majidi-Zolbadin et al., 2015). Instead, the behavioral and molecular effects of gestational exposure to stress on EAE course have not been investigated so far, and our work indeed represents the first study aiming to address this aspect.

Here we demonstrated that maternal stress during the last days of gestation worsens EAE outcome in the adult female offspring. Specifically, despite no differences in the weight or in nest building abilities were found between control and stressed mothers, we proved that gestational stress exerts long-lasting effects on the offspring by increasing susceptibility to EAE as indicated by the more severe symptoms scoring of prenatally stressed animals, which was statistically significant in both the acute and recovery phases of EAE course. Even though to our knowledge this is the first evidence concerning stress *in utero*, our behavioral results are in line with other studies reporting stress-related exacerbation of the disease at both behavioral and molecular levels. It has been demonstrated that chronic restraint stress during adulthood enhances demyelination in another MS animal model, the Theiler's murine encephalomyelitis virus (TMEV) infection (Young et al., 2010) and that chronic sound stress resulted in increased severity of neurological signs and histological lesions of the spinal cord in stressed EAE rats compared to the non-stressed ones (Núñez-Iglesias et al., 2010). Moreover, acute immobilization stress in adult animals shortens the time to EAE onset (Chandler et al., 2002).

Previous studies have shown that the EAE acute phase is characterized by an increased number of GPR17-expressing cells blocked at immature stages and not contributing to remyelination (Chen et al., 2009; Coppolino et al. 2018). Our data suggest that PNS exacerbates this defect, and that the stress-induced EAE severity may be due, at least in part, to reshaping of the remyelination process in the spinal cord, where more GPR17-positive immature oligodendrocytes were found. This effect has an important translational relevance since GPR17, a G protein-coupled receptor which is physiologically down-regulated after the immature oligodendrocyte stage, has been identified as an ideal target for new regenerative therapeutic approaches for MS and other myelin-associated disorders (Fancy et al., 2010; Fumagalli et

al., 2017; Lu et al., 2018). To further investigate the potential mechanisms underlying its modulation by prenatal stress, we focused our analyses on the Akt/mTOR signaling, since mTOR has a pivotal role in cell growth, differentiation and survival, and has been implicated in oligodendrocyte development and myelination as well as in GPR17 regulation (Tyler et al., 2009; Fumagalli et al., 2015). Moreover, preclinical studies indicate that, at cerebral level, this pathway is influenced by different stress paradigms (Chandran et al., 2012; Xia et al., 2016). In line with our assumptions, we observed a decrease of phosphorylated levels of mTOR as well as of the total form of Akt, the protein kinase known to stimulate mTOR activation. It is worth mentioning that the modulation of GPR17 by mTOR observed here is supported by results from a proteomic analysis revealing an increase of GPR17 in OPCs cultures treated with the mTOR inhibitor rapamycin (Tyler et al., 2011), an effect likely due to reduction of the G protein-coupled receptor kinase (GRK2) that could, in turn, prevent physiological GPR17 down-regulation via the key regulator of cell proliferation and apoptosis Murine Double Minute 2 (MDM2) (Fumagalli et al., 2015). Interestingly, the Akt/mTOR pathway is known to be activated by several growth factors, including the neurotrophin Brain-derived neurotrophic factor (BDNF) (Yoshii et al., 2011). The link between stress and BDNF is well-established (Molteni et al., 2016; Gray et al., 2013, McEwen et al., 2015, Calabrese et al., 2014), and different studies show that prenatal stress exposure can lead to BDNF alterations later in adulthood, both in the brain (Boersma et al., 2014;Yeh et al., 2012; Blaze et al., 2017; Luoni et al., 2014, 2015) and in the spinal cord (Winston et al., 2017). Furthermore, BDNF has a crucial role in cell growth and survival (Murray and Holmes, 2011) and several studies support the hypothesis of a neuroprotective function of this neurotrophin in myelination and myelin repair (Acosta et al., 2013; De Santi et al., 2009; Linker et al., 2010). Consistently, BDNF treatment

using transformed bone marrow stem cells reduces inflammation and apoptosis in EAE mice (Makar et al., 2008) and delays symptoms onset, reducing the overall EAE clinical severity (Makar et al., 2009).

It is important to note that, in our study, modulation of the Akt/mTOR pathway and the consequent increase in the number of GPR17-positive OPCs found in prenatally stressed EAE mice are paralleled by a reduction of BDNF. Specifically, the neurotrophin protein levels were reduced in the spinal cord of all mice subjected to EAE induction similarly to what observed for total BDNF mRNA levels, while the gene expression of the long 3' UTR BDNF form was down-regulated only in prenatally stressed mice. This form of BDNF represents the pool of transcripts localized at dendritic level thanks to the so-called "dendritic targeting" process (Tongiorgi et al., 2008), which occurs in an activity-dependent manner and enables the local synthesis of proteins required for neuronal development and plasticity, two features known to be altered by stress exposure (Wang et al., 2017). In line with this profile, we observed a similar modulation for BDNF isoform VI that belongs to this pool of transcripts and is localized in the distal dendrite (Baj et al., 2011). On the contrary isoform IV, spatially segregated in the soma/proximal portion of the dendrite, was broadly decreased in all EAE mice, suggesting that PNS may alter the proper dendritic targeting of BDNF transcripts, thus leading to a cascade of molecular events culminating in impaired maturation of OPCs and in more severe EAE symptomatology.

In conclusion, our study demonstrates for the first time that stress events occurring during the intrauterine life may exacerbate EAE clinical manifestations, giving new insights in the role of early life adversities in the etiopathogenesis of EAE/MS. Our data also indicate that PNS reshapes the process of remyelination in the spinal cord through an impairment of the AKT/mTOR pathway associated with a reduction of

BDNF levels. Since several already marketed drugs are able to modulate BDNF levels, the possibility of drug repositioning for multiple sclerosis should be addressed in future studies.

4.2 Long-term effect of prenatal stress exposure on acute responsiveness in adult mice: focus on inflammation and oxidative stress

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Manuscript in preparation

4.2.1 Introduction

During fetal life the body goes through a “critical” phase of development. Indeed, it is known adverse events during the prenatal period can result in long-lasting changes in adulthood (Darnaudéry and Maccari, 2008; Weinstock, 2005). These changes pertain to a wide spectrum of physiologic alteration: from epigenetic to inflammatory changes to dysregulation of the hypothalamic pituitary axis (HPA) axis. As a consequence, exposure to early life stressful events has been associated with behavioral alterations in adolescence and adulthood, such as aggression (Winiarsky et al., 2018), anxiety (Fonzo et al., 2016), hyperactivity and attention-deficit disorders (Bock et al., 2017), cognitive impairment (Pechtel and Pizzagalli, 2011) and increased incidence and susceptibility of major depressive disorders as well as of other psychiatric illnesses (Cattaneo and Riva, 2016). Moreover, experiencing stress during early stages of life may also increase the reactivity to subsequent stressors by leaving ‘scars’ of susceptibility that may facilitate improper and maladaptive response to further challenges. Indeed, prenatal stress has been demonstrated to exert a programming effect on sensitive neuronal brain networks related to the stress response, thus leading to enduring hyper- or hypo-activation of the stress system.

Therefore, aim of this study was to investigate the long-lasting consequences of stress *in utero* on the responsiveness to an acute challenge in adulthood, taking into account the immediate and the delayed molecular response. To do so, we exposed to an acute session of forced swim stress (6’) adult male mice born from dams that received daily sessions of restrain stress from gestational day 16 (GD16) until delivery. Our molecular analyses targeted the group of primary response genes (Immediate early genes) as well as mediators of the

inflammatory response and oxidative state and were performed in the mouse hippocampus 5 min or 2 h after the end of the stress.

4.2.2 Results

4.2.2.1 Behavioral Results

At first, we examined the effect of the prenatal stress on the body-weight gain of male pups, from post-natal day 24 (PND 24) to post-natal day 78 (PND 78).

4.2.2.1.1 Effect of prenatal stress exposure on the male progeny

Since a reduction of body-weight is a common effect of stress exposure, we monitored the body-weight of the pups between the weaning at post-natal day 24 (PND 24) and post-natal day 78 (PND 78). As shown in figure 7, pups exposed to the prenatal stress (PNS) showed a significantly lower body-weight with respect to control animals ($F_{1,57}$=8,742; P=0.0045 vs. CTRL). This effect was evident until PND 56 whereas at PND 78 the body-weight of the mice belonging the two experimental groups was similar.

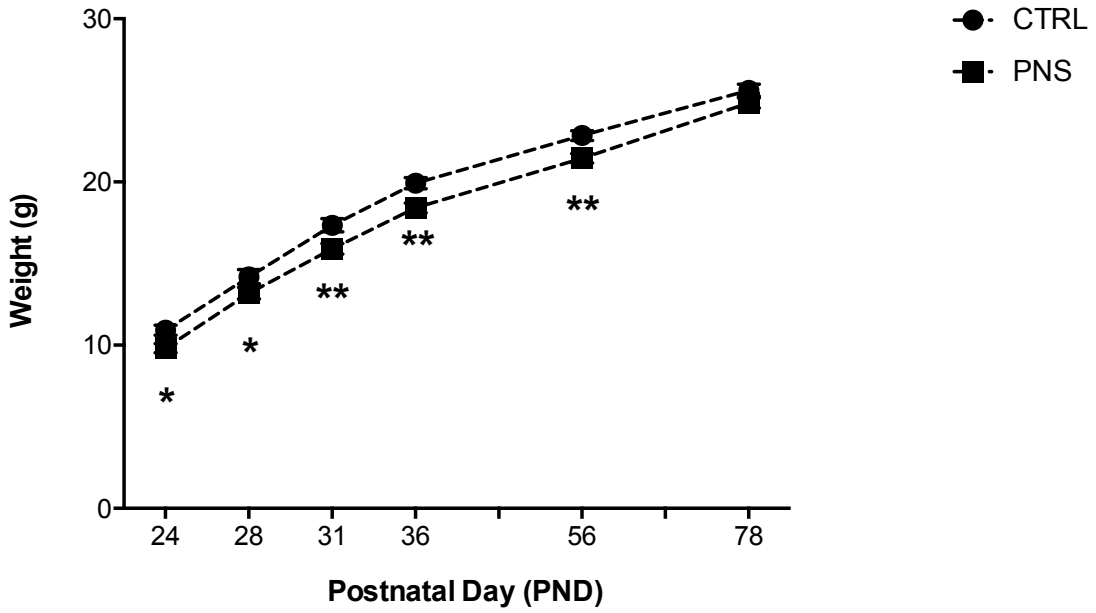

Fig.7 Effect of prenatal stress exposure on the pups

The body-weight of the animals has been monitored from PND 24 to PND 78. The data represents the mean ± SEM of independent determinations. *P<0.05, **P<0.01 vs. CTRL (Two-way ANOVA with Fisher's LSD).

4.2.2.2 Molecular Results

We then investigated if prenatal stress altered systems known to be altered in stress-related disorders. We conducted these analyses in the hippocampus, one of the brain region strongly involved in psychiatric disorders as well as stress response.

4.2.2.2.1 Effect of prenatal stress and acute stress on the expression of immediate early genes

As a first step, we evaluated the impact of our "double hit" experimental paradigm on the mRNA levels of the immediate early genes (IEGs) Activity-Regulated Cytoskeletal Associated Protein (Arc) and cFOS, as

markers of neuronal activation. Indeed, it is well established that the IEGs expression is transiently increased in response to neuronal activit triggered by several stimuli. Accordingly, we found that acute stress (AS) in adulthood significantly affected Arc expression ($F_{2,24}$=62.16; P<0.0001). Specifically, as shown in figure 8A, Arc mRNA levels were strongly up-regulated in the hippocampus of control animals after acute stress, an effect that was observed only at the earlier time point (t1) (+308%, P<0.0001 vs. CTRL/NO AS) whereas it returned to basal levels 2 hours later (t2). Interestingly, although prenatal stress (PNS) per se did not induce any change on Arc, it was able to influence the acute responsiveness of prenatally stressed mice, as indicated by a significant PNS x AS interaction ($F_{2,24}$=14.05; P<0.0001). Indeed, the increase of Arc expression previously observed in control animals right after the end of the AS was not detected in animals that were exposed to PNS.

Conversely, as shown in figure 8B, PNS did not affect the acute responsiveness in term of cFOS expression. Indeed AS significantly increased the mRNA levels of cFOS ($F_{2,25}$=37.98; P<0.0001) at the acute time point (t1) not only in control mice (+189%, P<0.0001 vs. CTRL/NO AS) but also in prenatally stressed mice (+121%, P<0.0001 vs. PNS/NO AS).

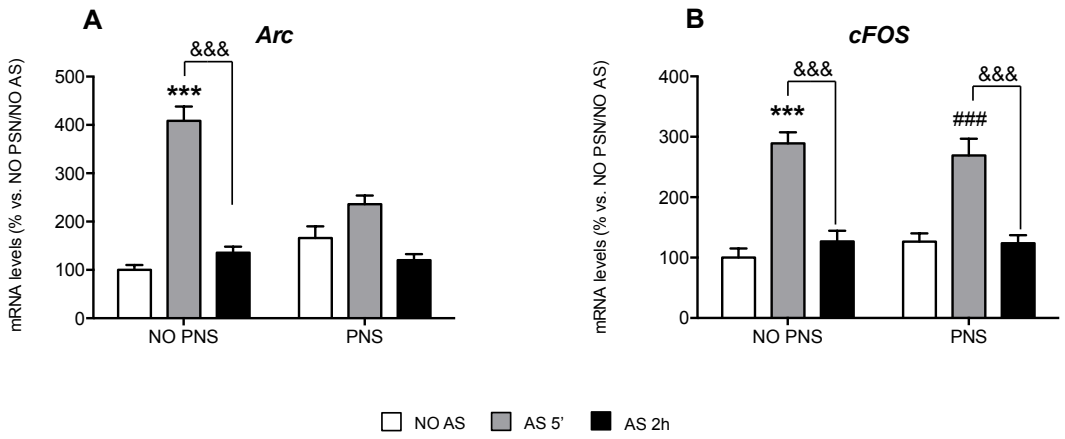

Fig.8 Modulation of IEGs gene expression by prenatal stress and acute stress

The mRNA levels of Arc (A) and cFOS (B) were measured in the hippocampus of control (CTRL) and prenatally stressed (PNS) mice exposed in adulthood to an acute stress (AS). The analyses were performed 5 minutes (t1) or 2 hours (t2) after the end of the stress in comparison with control unstressed animals (CTRL/NO AS). The data, expressed as percentage of CTRL/NO AS animals (set at 100%), are the mean ± SEM of independent determinations. ***P<0.0001 vs. CTRL/NO AS; $^{###}$P<0.0001 vs. PNS/NO AS; $^{&&&}$P<0.0001 vs. AS (Two-way ANOVA with Fisher's LSD).

4.2.2.2.2 Effect of prenatal stress and acute stress on the expression of inflammatory cytokines

Since stress-related disorders are known to be associated with alterations of the inflammatory system and stressful events affects the levels of specific mediators of the inflammatory response, we analyzed the expression of pro- and anti-inflammatory cytokines in our experimental paradigm. The release of cytokines may represent the first response of the immune system to cope an acute stress. Accordingly,

we analyzed the mRNA expression of IL-1β, IL-6 and TGF-β, and the protein levels of IL-10.

As shown in figure 9A, despite the large variability, we found a significant effect of PNS on IL-1β gene expression ($F_{1,25}$=4.79; P<0.05). Specifically, the mRNA levels of the cytokine were increased in the hippocampus of prenatally stressed mice (+95%, P<0.05 vs. CTRL/NO STRESS). Conversely, we did not observe any changes of IL-1β after acute stress, although the up-regulation of the cytokine detected in prenatally stressed animals was significantly lower 2 hour after the exposure to the acute stress (-48%; P<0.05 vs. PNS/NO AS), as indicated by the statistical analysis ($F_{2,25}$=4.871; P<0.05).

Next, we evaluated the impact of our paradigm on the mRNA levels of IL-6 (Fig.9B) finding a similar profile in both control and prenatally stressed mice. Specifically, the expression of this cytokine was not affected by the prenatal stress but it was altered in response to the acute stress ($F_{2,30}$=5.357; P<0.05). In particular, IL-6 was up-regulated right after the end of the AS (t5') in both control (+45% vs. CTRL/NO AS, P<0.05) and prenatally stressed animals (+47% vs. PNS/NO AS, P<0.05). This effect persisted 2 hours after the end of the stress, although it was significant only in PNS mice (+41% vs. PNS/NO STRESS, P<0.05).

We then decided to investigate the modulation of the anti-inflammatory cytokines TGF-β andIL-10 (Fig.9C, D). The Two-way ANOVA analysis of TGF-β gene expression revealed a significant effect of PNS ($F_{1,25}$=13.52; P<0.01). Indeed, PNS animals exposed to the second challenge, displayed lower levels of the cytokine at both time points compared to control mice (-67% vs. CTRL/AS 5', P<0.01; -37% vs. CTRL/AS 2h, P<0.01 respectively). Regarding IL-10, we found a trend to increase in control mice in response to the AS that reached a peak 2 hours after the end of the stress (+30% vs. CTRL/NO AS). This modulation was not observed in PNS animals, which displayed reduced

levels of IL-10 in basal condition (-32% vs. CTRL/NO AS), an effect that did not reach the statistical significance despite a main effect from PNS ($F_{1,18}$=19.27; P<0.001).

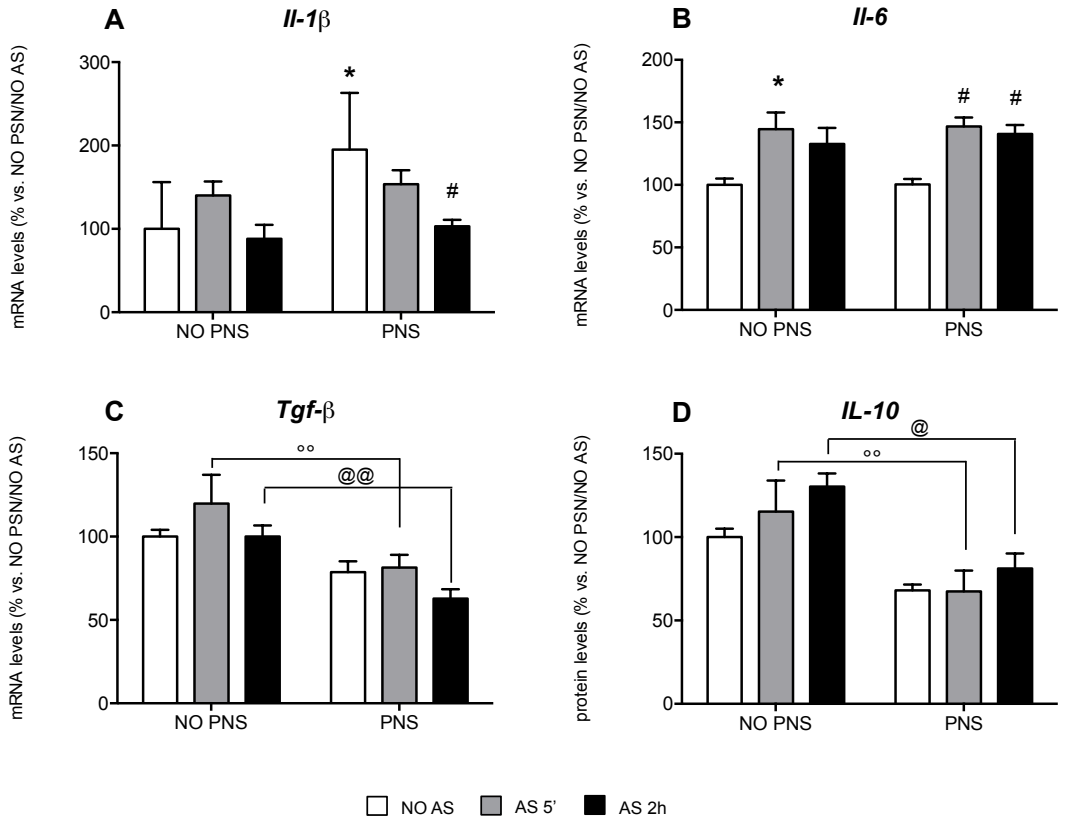

Fig.9 Modulation of pro- and anti-inflammatory cytokines by prenatal stress and acute stress.

The mRNA levels of the pro-inflammatory cytokines IL-1β (A), IL-6 (B) and TGF-β (C) and the protein levels of anti-inflammatory cytokine IL-10 (D) were measured in the hippocampus of control (CTRL) and prenatally stressed (PNS) mice exposed in adulthood to an acute stress (AS). The analyses were performed 5 minutes (t1) or 2 hours (t2) after the end of the stress in comparison with control unstressed animals (CTRL/NO AS). The data, expressed as percentage of CTRL/NO AS animals (set at 100%), are the mean ± SEM of independent determinations. *P<0.05 vs. CTRL/NO AS; $^{\#}$P<0.05 vs. PNS/NO AS;

°°P<0.01 vs. CTRL/AS t1; @P<0.05, @@P<0.01 vs. CTRL/AS t2 (Two-way ANOVA with Fisher's LSD).

4.2.2.2.3 Effect of prenatal stress and acute stress on mediators of the oxidative balance

Lastly, we analysed the potential impact of prenatal stress and its influence on the effect of the acute challenge focusing on mediators of the redox balance. The modulation of the redox balance plays a key role in response to several acute and chronic stimuli. In stress conditions, specific anti-oxidant mechanisms are activated in order to rapidly restore the right balance in the cell and prevent the damage caused by the high cytotoxicity of pro-oxidant molecules. Accordingly, we investigated the expression of both pro- and anti-oxidant mediators and enzymes and in particular, we analysed the expression of the anti-oxidant mediators Nrf2, Srxn1 and Prx-SO3 and that of the pro-oxidant enzyme Nox2.

We first measured the protein levels of Nrf2 in the nuclear component, where it acts as transcription factor for several anti-oxidant enzymes. We found that its levels were not significantly affected by PNS under basal conditions, however the prenatal paradigm influenced the response to the acute challenge, as indicated by a significant PNS x AS interaction ($F_{2,24}$=4,361; P<0.05). Specifically, as shown in figure 10A, Nrf2 was up-regulated by AS in both control and PNS mice at the earlier time point, an effect that reached the statistical significance only in PNS mice (+65%, P<0.05 vs. PNS/NO AS). In this experimental group, this increase was even higher 2 hours after the end of the challenge (+105%, P<0.01 vs. PNS/NO AS), whereas it did not persist in control mice.

Next, we assessed the mRNA levels of sulfiredoxin1 (Srxn1), one of the enzymes whose transcription is regulated by Nrf2. The analyses revealed that Srxn1 was modulated by both PNS ($F_{1,25}$=7.739; P<0.05) and AS ($F_{2,25}$=4.011; P<0.05). In particular, as shown in fig. 10B, we observed a trend to decrease of its gene expression after PNS in basal conditions (-30% vs. CTRL/NO AS) whereas the enzyme was up-regulated after AS. The effect of the acute challenge was observed in both control and prenatally stressed mice, however only in the latter experimental group it reached the statistical significance; probably due to the lower levels observed in PNS mice not exposed to AS.

We then examined the level of the over oxidized enzyme Prx-SO3 (Fig.10C) finding no changes by the prenatal stress paradigm but a significant modulation by the acute stress ($F_{2,18}$=4.473; P<0.05) and a PNS X AS interaction ($F_{2,17}$=4.733; P<0.05). More in detail, the protein levels of Prx-SO3 were slightly up-regulated by the acute challenge in both control (+34% vs. CTRL/NO AS) and prenatally stressed mice (+40% vs. PNS/NO AS) 5 minutes after the end of the stress, although this effect was not statistically significant. This trend in increase was not observed at the longer time point in control mice, while it was even higher in PNS animals (+116% P<0.01 vs. PNS/NO AS).

Moreover, we assessed the gene expression of the pro-oxidant enzyme Nox2 (Fig.10D). Nox2 mRNA levels were not statistically affected by PNS, even though a trend can be observed. Nox2 mRNA levels tend to decrease in control mice exposed to acute stress, while are higher in PNS animals at the longer time point (+49%; P<0.05 vs. CTRL/AS 2h).

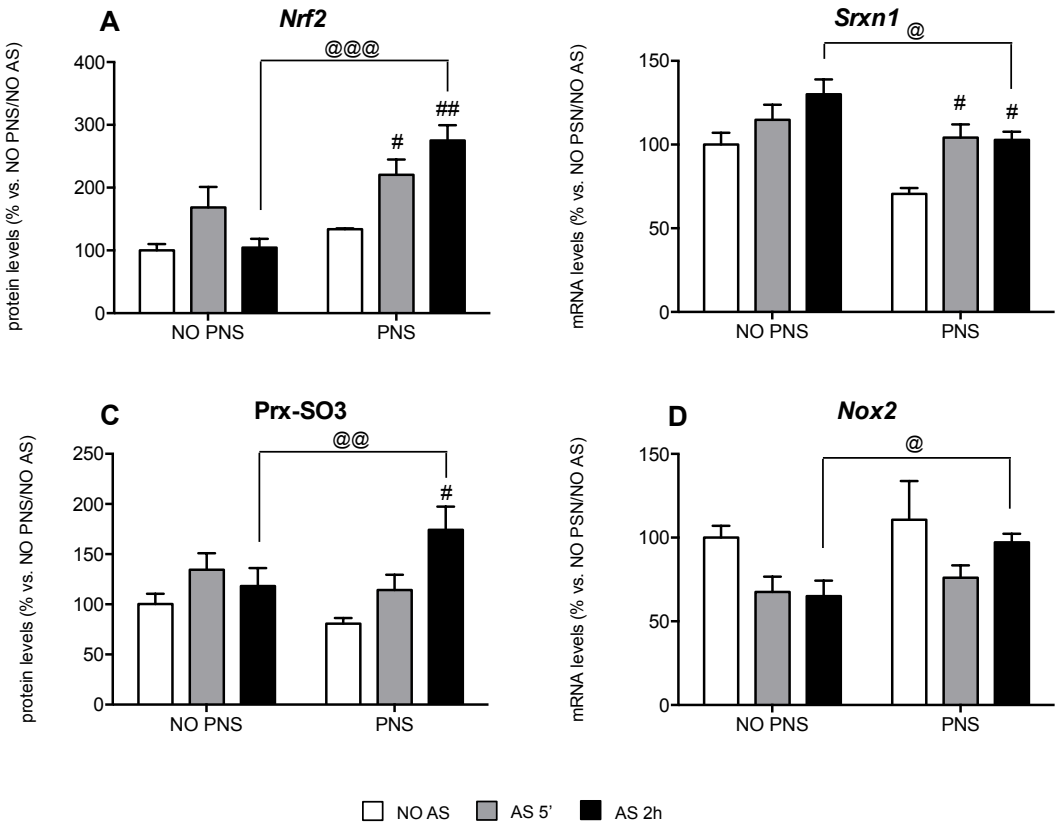

Fig.10 Modulation of modulators of the redox balance by prenatal stress and acute stress

The nuclear protein levels of Nrf2 (A), the mRNA levels of Srxn1 (B), the protein levels of PRX-SO3 in the total homogenate (C) and the mRNA levels of Nox2 were measured in hippocampus of control (CTRL) and prenatally stressed (PNS) mice exposed in adulthood to an acute stress (AS). The analyses were performed 5 minutes (t1) or 2 hours (t2) after the end of the stress in comparison with control unstressed animals (CTRL/NO AS). The data, expressed as percentage of CTRL/NO AS animals (set at 100%), are the mean ± SEM of independent determinations. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. PNS/NO AS; $^{@}P<0.05$, $^{@@}P<0.01$,$^{@@@}P<0.001$ vs. CTRL/AS t2 (Two-way ANOVA with Fisher's LSD).

4.2.3 Discussion

The present study demonstrates that the exposure to a prenatal stress paradigm not only has long-term effects on the inflammatory and oxidative balance in the hippocampus of adult mice under basal conditions, but it is also able to interfere with their responsiveness to a challenging situation such as an acute stress. Based on the etiological role of early-life stressful experiences for psychiatric disorders, our results support the idea that this may occur through the impairment of specific molecular systems crucial for a proper coping with challenging situations.

It is well established that stress represents the main environmental risk factor for the development of several psychiatric disorders such as major depression disorder, schizophrenia and bipolar disorder. Particularly, given the importance of perinatal periods for brain development, stressful events occurring during CNS development may induce long-term brain structural and functional alterations, that could enhance the vulnerability to mental disorders later in life (Kim et al., 2015; Markham and Koenig, 2011). These brain abnormalities reflect the impairment of neuroplasticity mechanisms that characterize psychiatric disorders and that may be defined as the brain's capacity to alter its structure and function in reaction to environmental stimuli.

For this project we decided to use a "double hit model" to investigate the long-term impact of prenatal stress in mice on molecular mediators of the cerebral inflammatory and oxidative state known to be altered in psychiatric disorders. Specifically, we analysed the effects produced by prenatal stress in adulthood in both basal conditions and after the exposure to an acute challenge in male mice. To this aim, we exposed pregnant mice to daily sessions of restraint stress during the last gestational week, and the male progeny to an acute forced swim stress in adulthood.

At first, we found that stress reduced the body-weight gain of the male pups after the weaning until adulthood. This result is in line with preclinical (Lesage et al., 2004) and clinical studies where in addition stress-induced low birth weight was associated with long-term reduction of the cortical surface area and impaired cognition (Wainstock et al., 2014; Walhovd et al., 2016). These data demonstrated that our prenatal stress paradigm had an impact on the progeny, suggesting possible alterations also at molecular level. Moreover, since stress affects neuroplasticity, which is a “dynamic” concept, we decided to evaluate potential long-term molecular changes not only in basal conditions but also in response to an acute challenge.

As a first step, we decided to evaluate if prenatal stress could affect the induction of immediate early genes (IEGs), an index of neuronal activation, which occurs in response to an acute challenge. The synthesis of the IEGs occurs within minutes and play important roles in several cellular processes involved in synaptic plasticity, LTP, and LTD (long-term potentiation and long-term depression respectively), thus influencing the function of various neural circuits (Loebrich and Nedivi, 2010). As expected, the expression of the IEG Arc and cFOS was increased following the acute stress at the earlier time point, however, this effect was not observed for Arc in the hippocampus of prenatally stressed mice. Arc mRNAs are known to undergo a rapid transport to dendrites and a local synaptic translation. The protein synthesized is then required for synaptic plasticity, memory consolidation, LTP and LTD, processes that involve multiple neurotransmitters such as glutamate and dopamine and their receptors (Chowdhury et al., 2006; Plath et al., 2006).

Among the molecular processes altered in psychiatric diseases and known to be a target of stress, we investigated the inflammatory response, which also represents one of the primary reactions to an acute challenge. Interestingly, we found increased expression of the

pro-inflammatory IL-1β in prenatally stressed animals, in accordance with other studies (Diz-Chaves et al. 2013; Ślusarczyk et al., 2015) and we hyphotesized that the induction of a pro-inflammatory state by prenatal stress could also impair the ability of the animals to respond to subsequent challenges, contributing to behavioural alterations. Since increased level of IL-1β has been linked to psychiatric diseases such as depression, schizophrenia and bipolar disorder, the prenatal stress-induced up-regulation of this cytokine could be one possible molecular mechanism for the stress-related development of depressive-like and other pathological behaviours, as it has already been proposed in animal models (Kubera et al., 2011).

In parallel, we found that prenatal stress reduced the levels of the anti-inflammatory cytokine IL-10. Changes in IL-10 have been associated with depressive symptoms in humans (Song et al., 2009) and are proposed to influence depressive behaviour. For example, it has been reported that chronic restraint stress induced a long-lasting decrease of hippocampal IL-10 mRNA and circulating protein levels in animals showing depressive-like behaviour (Voorhees et al., 2013). Moreover, IL-10 administration before performing a forced swim test was able to reverse the chronic stress-induced depressive phenotype, supporting a role for IL-10 in affecting behaviour.

Alterations of inflammatory response may have several consequences, among them, changes in the redox status, which is known to occur also in psychiatric disorders (Smaga et al., 2015). At first, we decided to evaluate the functioning of the anti-oxidant machinery, analysing one of the nuclear factor E2-related factor 2 (Nrf2) pathways. Nrf2 is a transcriptional factor known to translocate in the nucleus under oxidative stress conditions, and through the binding of the antioxidant responsive element (ARE) is able to activate the transcription of many different anti-oxidant factors. Among those factors there is Sulfiredoxin1 (Srxn1), a small enzyme that catalyses the reduction of hyper-oxidized

peroxiredoxins (Prxs), allowing them to be reactivated to function again as a reducer of H_2O_2 in H_2O using the reducing power of glutathione (GSH) and thioredoxin (Trx) (Soriano et al., 2008; Sunico et al., 2016). In our study, we observed that while in control animals the acute stress was able to induce a slight increase in the translocation of Nrf2 into the nucleus that disappeared after 2 hours, in prenatally stressed animals, the increase of the nuclear Nrf2 was greater and amplified two hours after the acute stress exposure. The increased expression of nuclear Nrf2 could be the reaction to an over-production of free radicals and reactive species in prenatally stressed animals after a stimulus, as indicated in studies showing increased and persistent translocation of Nrf2 into the nucleus in situation of oxidative stress (Cui et al., 2016). In line with the increased nuclear levels of Nrf2, we observed that acute stress up-regulated Srxn1 in both controls and prenatally stressed mice, although only in prenatally stressed animals this increase reached the statistical significance. Nevertheless, the up-regulation of Srxn1 seemed to be efficient only in control animals, in which, after a slight increase in the protein levels of Prx-SO3 right after the acute stress, the levels of the hyper-oxidized protein returned to basal levels. By contrary in prenatally stressed animals, the production of Prx-SO3 kept rising two hours after the end of the acute stress, suggesting once again an imbalance in the oxidative state of those animals, which could be due to a greater production of reactive species or to a malfunction in their anti-oxidant machinery.

We then examined a pro-oxidant factor, the NADPH oxidase 2 (NOX2), whose function is to generate ROIs (Reactive Oxidant Intermediates), able to damage DNA, proteins and lipids, and usually activated in order to prepare the brain for a pathogens attack. We observed a gradual decrease in the mRNA levels of this enzyme in the acutely stressed animals, effect that was not maintained in prenatally stressed groups. Spiers et al. found a similar result, with a down-regulation of NOX2 after

an acute restraint stress (Spiers et al., 2016), suggesting that this reduction might be due to the activation of the peroxisome proliferator-activated receptor.

In conclusion, from this study emerged that prenatal stress is able to modify the basal levels of specific inflammatory and oxidative mediators, but it is also capable to influences the response of these systems in reaction to an acute challenge. Moreover, our results highlight that the influence of the prenatal stress on the acute responsiveness was stronger 2 hours after the challenge. This finding suggests that the recovery after an acute challenge is in some way influenced by prenatal stress, and that the physiological reestablishment of homeostasis is impaired by the exposure to early-life adversities.

4.3 Influence of pre-exposure to social defeat stress on repetitive mild traumatic brain injury outcome in adolescent mice

Maria Serena Paladini, Karen Krukowski, Susanna Rosi and Raffaella Molteni

Unpublished data

4.3.1 Introduction

Adolescence, the period marking the transition from childhood to adulthood, is frequently marked by age-specific behavioral phenotypes, such as increased emotional reactivity, social activity, playfulness and risk-seeking behavior (Casey et al., 2008). This is typically coincident with changes in the social environment, such as spending more time with peers and less with parents. Together with the neurobiological changes characteristic of puberty, these age-specific behavioral features may enhance the susceptibility to environmental factor, such as stress. Indeed, not only the nature of stressors changes during adolescence, but also the mechanisms the adolescent body orchestrates to respond to stress. Supporting this, animal studies indicated that an equivalent dose of corticosterone increased gene expression to a greater degree in the adolescent compared to adult hippocampus (Lee et al., 2003). Moreover, it is well recognized that the brain areas known to be the most sensitive to stress in adulthood, namely the hippocampus, prefrontal cortex, and amygdala, are still under development during adolescence (Giedd and Rapoport, 2010). Thus, the convergence of all these factors may render the adolescent brain especially sensitive to external adverse perturbations, and therefore more vulnerable to physical and psychological morbidities (Romeo and McEwen, 2006).

With these premises, in this project I deepened how experiencing stress during adolescence may have repercussions on a further external insult extremely frequent during this time of life, such as brain concussion. Indeed, because of their usual involvement in sports and higher-risk activities, adolescent sustain the majority of sports-related brain injuries (Semple et al., 2015). Traumatic brain injury (TBI), defined as an insult to the brain from an external mechanical force, is known to produce both acute and chronic consequences that lead to permanent disabilities. Indeed, the direct consequences of a single or repetitive

concussions can result in various secondary pathological conditions, including seizures, sleep disorders, neurodegenerative diseases, neuroendocrine dysregulation, and psychiatric problems (Bramlet and Dietrich, 2015). Despite stress-related disorders as a consequences of TBI have been largely investigated (Bryant, 2011), the long-term repercussions of early-life adversities –specifically during adolescence- on behavioral and molecular outcomes after TBI have not been examined yet.

For this reason, on postnatal days (PND) 28, I exposed adolescent C57Bl6 male mice to 10 days of repeated social defeat stress. The social defeat paradigm possesses higher face, predictive, and ethological validity that results in enduring behavioral and neurobiological changes that mimic several symptoms of the human condition such as decreased preference for sucrose and increased social avoidance, behaviors collectively described as a depressive-like phenotype (Krishnan and Nestler, 2008; Berton et al., 2006). Four days after the end of the stress, at PND 42, the mice received 2 mild concussions, 24h apart from each other, using the Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) apparatus. The mice were then assessed for anxiety-like behavior in the elevated plus maze and learning and memory in the novel object recognition (NOR) test.

I carried out this experiment during the 6 months I spent as a visiting PhD student at the laboratory of Brain Injury, Neuroinflammation and Cognitive Function headed by Professor Susanna Rosi at the University of California, San Francisco.

4.3.2 Results

4.3.2.1 Impact of previous stress exposure on 2rTBI behavioral outcome

Starting from one week after injury (dpi 7) at PND 49 mice were tested in the elevated plus maze (EPM) and at PND 64 (dpi 21) for the novel object recognition test.

To examine if 2rTBI mice were influenced by previous stress exposure in developing anxiety-like and security seeking behavior, we evaluated these phenotypes using the elevated plus maze in which mice freely explore either two closed, dark arms or two open, illuminated arms. The Two-way ANOVA analysis showed a significant effect of stress exposure ($F_{1,42}$=8.447; P<0.01). Indeed, defeated mice spent a significantly shorter time in the open compared to the sham not stressed animals, although this effect is statistically significant only in stressed sham animals (-33% vs. No Stress/sham, P<0.01) (Fig. 11A).
We then test the mice for hippocampal-dependent memory impairment using the NOR test. As depicted in figure 11B, we observed a significant Stress x 2rTBI interaction ($F_{1,48}$=10.26; P<0.01). More in detail, RSDS mice displayed no preference toward either object, resulting in a lower discrimination index compared to not stressed sham group (P<0.05 vs. No Stress/sham). 2rTBI mice as well showed a tendency, although not significant, in a lower discrimination index. Interestingly, mice that were exposed to the combined paradigm, exhibited an increased preference toward the novel object, with a higher discrimination index both compared to the stressed sham counterpart (P<0.001 vs. RSDS/sham) and to only injured animals (P<0.05 vs. No Stress/2rTBI).

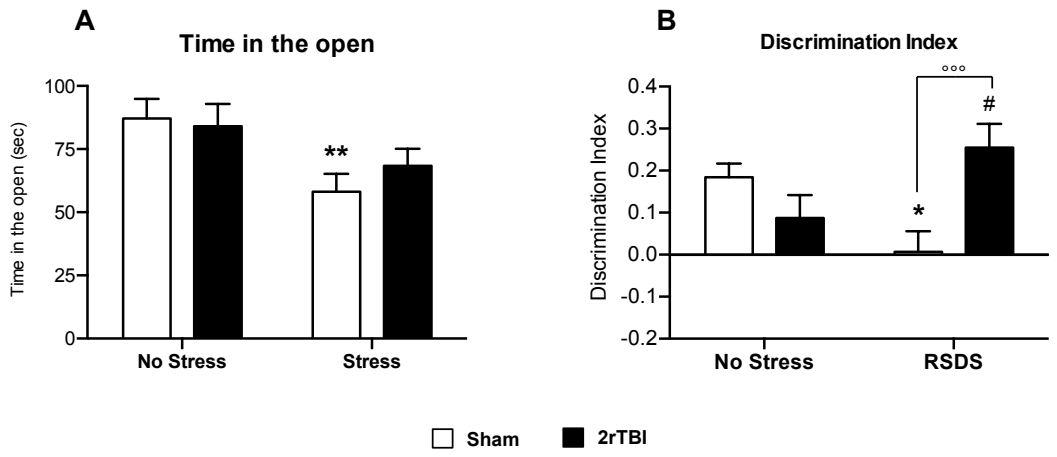

Fig.11 Impact of previous stress exposure on 2rTBI behavioral outcome
Mice were tested in the EPM at PND 49 and the time in the open areas of the maze was analyzed (A). At PND 64, the hippocampal-dependent recognition memory were tested using the NOR paradigm and the discrimination index (B) was calculated as described above. For all the analyses the data are expressed as mean of the examined variable ± SEM of independent determinations. *P<0.05, **P<0.01 vs. NoStress/sham; $^{\#}$P<0.05 vs. No Stress/2rTBI; $^{\circ\circ\circ}$P<0.001 vs. RSDS/sham (Two-way ANOVA with Fisher's LSD).

4.3.2.2 Analyses of Iba1 immunostaining in the hippocampus of mice exposed to social defeat stress and 2rTBI during adolescence

Microglia cells are the macrophagic resident cells of the brain, responsible of the first line defense against immune alterations within the brain. In the so-called "resting state" microglia surveil the microenvironment searching for pathogens or signs of disuse damage. At this stage microglia have a dendritic morphology characterized by long and ramified processes. In the "activated state" microglia have an amoeboid form with high mobility toward the site of damage. Microglia activation has been detected after exposure to social defeat stress in

different brain areas (Stein et al., 2017) as well as in animals that received different models of TBI (Donat et al., 2017).

To investigate microgliosis in the hippocampus, we quantified the density of Iba-1-positive cells in the CA1 region. As depicted in figure 12 we didn't observe any difference in the % of Iba1 staining between the different experimental groups.

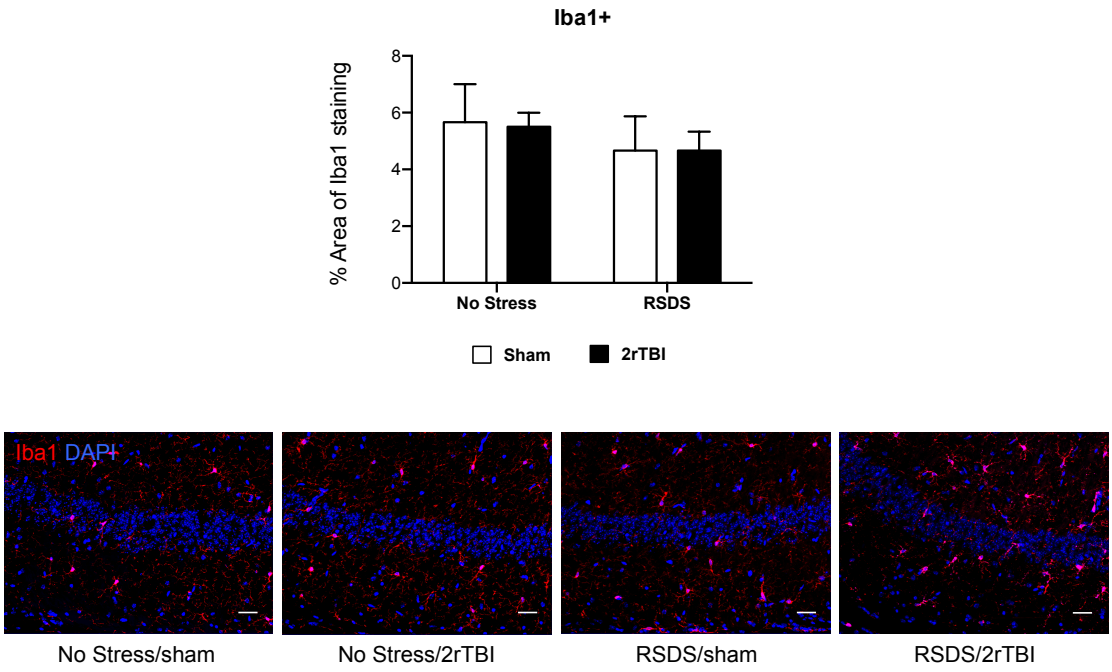

Fig.12 Analyses of Iba1 immunostaining in the CA1

% of Iba1 staining was measured in the CA1 region of the hippocampus of mice exposed to social defeat stress and 2rTBI during adolescence. Representative images of Iba1 staining showing Iba1 staining in red and DAPI in blue. Scale bar = 50 µm

4.3.3 Discussion

All aspects of life require stress. However, the type of stressors we face and how we respond to them change throughout our life. Adolescence represents a period of life when both the type of stressors and the adaptation mechanisms orchestrated by the body are changing. Indeed, compared to other life phases, the stressors that adolescents have to face are mainly of a social character. Social stress can take place either in the home environment or during the increasing amount of time that adolescents spend with peers, when bullying behaviors are extremely frequent (Buwalda et al., 2011). In this respect, negative social experiences during adolescence are known to increase the risk of psychiatric disorders in adulthood (McCormick, Green 2013). Another peculiar feature of adolescence is the increased risk of experiencing injuries, due to the fact that adolescents are more likely than adults to participate in organized sport but also to engage in risky and impulsive behaviors.

In this study I investigated if a previous exposure to a social stress could alter the behavioral and molecular outcome after experiencing concussions, a brain injury extremely frequent during adolescence (Semple et al., 2015). I took advantage of the repeated social defeat stress paradigm, a well-recognized model of psychological and social stress that is able to induce depressive-like phenotypes at preclinical level (Golden et al., 2011) and of a recently developed model of TBI, the Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) that has been only partially characterized. I firstly investigated how this combined experimental model could affect two behavioral features that are known to be altered by stress and TBI, such as anxiety-like/security seeking behavior and memory. Using the elevated plus maze I assessed and deepened the stress-induced anxiety-like phenotype and the injury-induced risk-taking behavior. Indeed, while stress exposure is known to reduce the time spent in the

open areas of the maze (Hata et al., 2001), repetitive mild concussions (n=5) using the CHIMERA apparatus have been demonstrated to have an opposite effect, suggesting post-injury behavioral disinhibition and increased risk-taking phenotype (Nolan et al., 2018). In my experimental settings, socially defeated mice displayed an anxiety-like phenotype, spending less time in the opens regardless of the concussions. On the other hand, TBI mice that received two mild concussions didn't display the risk-taking behavior. Since this specific injury model is very recent, there are no evidences about the effect of two mild concussions on the EPM task, either in adult or in adolescent animals, and therefore this is the first time, to the best of our knowledge, that this aspect is evaluated. However, dissecting if the observed effect was due to the age of the mice or the number of injuries needs still to be elucidated. I next investigated if stress and/or TBI could trigger hippocampal-related memory deficits using the NOR test. I observed a reduction of the discrimination index (a parameter that indicates the preference for the novel object) in mice that were exposed to RSDS and in non stressed animals that received the two mild concussions. This result is in line with other studies that demonstrated stress-induced (Patki et al., 2013) and trauma-induced (Bodnar et al., 2019) memory impairment. However, in this experiment, stressed mice that received the concussions didn't show any impairment. A possible explanation to this interaction could be that the combined condition rendered this group of mice overactive. Therefore, the observed data could result from a generally increased exploring time.

The employed version of the NOR consisted in 24 hours between the exposure to two identical objects and the novel one, thus testing hippocampal memory. Therefore, the molecular analyses were focused on this brain area, sensitive to stress exposure (Kim et al., 2015).

The analysis of the % of Iba1+ area carried in the mice CA1 didn't show any significant difference between the different experimental conditions,

suggesting that the observed behavioral results are not attributable to differences in microglia activation.

The obtained results represent the first evidences of a possible environmental role of previous social stress on TBI outcome during adolescence. Nevertheless, further investigations are needed to better characterize this new experimental model.

4.4 Long-lasting impact of chronic stress on hippocampal oxidative responsiveness to an acute challenge: effect of the antipsychotic lurasidone

Maria Serena Paladini, Andrea Carlo Rossetti, Vittoria Spero, Mariusz Papp, Paola Stella Brivio, Francesca Calabrese, Marco Andrea Riva and Raffaella Molteni

Manuscript in preparation

4.4.1 Introduction

The role of stress in the etiology of mood disorders has been widely investigated. Major depressive disorder (MDD) is the commonest cause of disability and affects nearly 16% of the global population (Kessler et al., 2003), however despite the large interest in improving the available therapeutic approaches, the pharmacological treatment still presents some critical issues, such as the high rate of resistance (Al-Harbi, 2012), the long latency of the therapeutic effect (Machado-Vieira et al., 2010) and the highly frequent occurrence of relapses, with at least 50% of those who recover from a first episode of depression having one or more additional episodes in their lifetime (Burcusa and Iacono, 2007). Indeed, while it is expected that antidepressant or other psychotropic drugs used in the treatment of depression may prevent relapse, little is known on how long-term pharmacological treatments properly work to manage the chronic course of the pathology and to maintain their clinical efficacy. Exposure to stressful events during adult life may leave long-term signature and increase the response to subsequent stressors. Indeed, it is possible that not all the systems impaired by stress are restored during the remission of the symptoms, thus leaving ‘scars’ of vulnerability that may facilitate the relapse to the pathology. Therefore, the main purpose of this part of my project was to investigate if and how stress-induced changes during adulthood may persist after a recovery period and to understand the molecular mechanisms that may underlie the precipitation of a recurrent episode. Moreover, we aim to establish whether such changes can be modulated by the treatment with the antipsychotic lurasidone, to gain insights its potential ability to prevent or reduce the individual susceptibility to subsequent negative events.

To address these objectives, adult male rats were exposed to four weeks of chronic restraint stress and, starting from the second week, received either vehicle or lurasidone chronically. The animals were then left undisturbed for the subsequent three weeks. Furthermore, after this

window of recovery, the rats were subjected to an acute immobilization stress. We focused our analyses on regulators of the oxidative balance, increasingly recognized to have a crucial role in the etiology of psychiatric disorders (Fraunberger et al., 2016), in the hippocampus, a brain region primary involved in the stress response and in the integration of information for past with present stimuli (Borders et al., 2017).

4.4.2 Results

4.4.2.1 Effect of acute stress exposure on the gene expression of the oxidative regulators Nox2 and Nrf2 after 3 weeks of washout from CRS and lurasidone treatment

Our laboratory has previously demonstrated that lurasidone is able to counteract the behavioral depressive-like phenotype induced by chronic stress by acting on key players in the maintenance of the oxidative balance in the brain, such as NADPH oxidase 2 (Nox2), an enzyme responsible for the production of reactive oxygen species (ROS), and the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor involved in the control of the cellular anti-oxidant response (Rossetti et al., 2018). In my PhD project I investigated if the modulation of these genes by chronic stress and lurasidone treatment could still be observed after a washout period. Moreover, we examined the possibility that previous chronic restraint stress (CRS) exposure and/or chronic lurasidone treatment may sensitize these systems to further environmental challenges, such as an acute stress.

As depicted in figure 13A and B, despite some tendencies, we did not observe any significant change in the mRNA levels of Nox2 attributable to CRS and lurasidone three weeks after the end of the stress in the dorsal and ventral portion of the hippocampus. Regarding the effect of acute stress, while we couldn't find any significant alteration in the dorsal hippocampus, in the ventral hippocampus we found that Nox2 gene expression was reduced after the acute stress only in rats treated with lurasidone compared to the not treated control and to the treated control without the acute challenge (-32% vs. No CRS/VEH/No AS, $P<0.05$; -31% vs. No CRS/LUR/No AS, $P<0.05$) (Fig.13B).

When we investigated the antioxidant counterpart, we did not find any significant modulation in both the areas analysed. However, in the ventral hippocampus, we observed a tendency toward a decrease of

Nrf2 in response to acute stress in both control (-14% vs. No CRS/VEH/NoAS, P>0.05) and chronically stressed animals (-14% vs. CRS/VEH/No AS, P>0.05), not present when the animals were treated with lurasidone (Fig.13D).

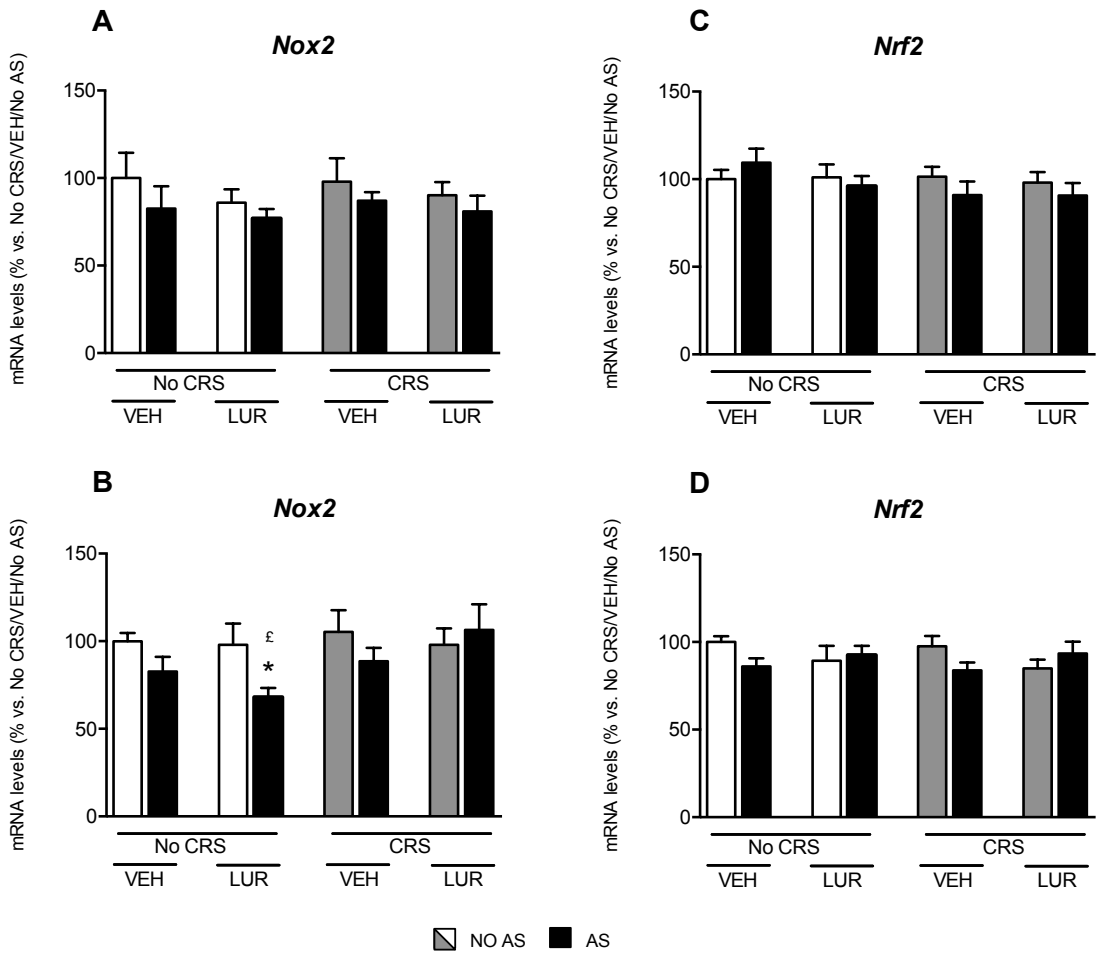

Fig.13 Effect of acute stress exposure on the gene expression of Nox2 and Nrf2 after 3 weeks of washout from CRS and lurasidone treatment

The mRNA levels of Nox2 and Nrf2 were measured in the dorsal (A, C) and ventral (B, D) hippocampus of not stressed (No CRS) and stressed (CRS) rats treated with vehicle (VEH) or lurasidone (LUR) and exposed after 3 weeks of washout to acute stress (AS) or not (No AS). The data, expressed as percentage of No CRS/VEH/No AS animals (set at 100%), are the mean ± SEM of independent determinations. *P<0.05 vs. No CRS/VEH/No AS; $^{£}$P<0.05 vs. No CRS/LUR/No AS (Three-way ANOVA with Fisher's LSD).

4.4.2.2 Effect of acute stress exposure on the gene expression of the antioxidant enzyme Srxn1 after 3 weeks of washout from CRS and lurasidone treatment

We next analyzed one of the detoxifying enzymes whose transcription is mediated by Nrf2, i.e. sufiredoxin1 (Srnx1). Regarding the dorsal hippocampus, the Three-way ANOVA analysis depicted a significant CRS x treatment x AS interaction ($F_{1,60}$=6.829, P=0.0113). More in detail, we found that acute stress significantly increased the gene expression of this antioxidant enzyme in rats not exposed to CRS treated with vehicle (+38% vs.No Stress/VEH/NoAS, P<0.05) (Fig.14A). Interestingly, Srxn1 up-regulation was not found in animals exposed to CRS and treated with vehicle (-33% vs. No CRS/VEH/AS, P<0.01), which showed instead a slight decrease compared to CRS rats not exposed to the acute challenge (-10% vs. CRS/VEH/No AS, P>0.05). It is noteworthy that lurasidone, when given to CRS rats, elicited an almost significant restorative effect on Srxn1 modulation after acute stress (+30% vs. CRS/LUR/No AS, P=0.07).

The Three-Way ANOVA analysis of Srxn1 modulation in the ventral hippocampus highlighted a stronger effect of acute stress ($F_{1,69}$=28.57, P<0.0001). Indeed, the up-regulation of Srxn1 induced by acute stress –compared to sham animals- is statistically significant in the not treated group (+37% vs. No CRS/VEH/No AS, P<0.001), in the group of animal treated with lurasidone (+28% vs. No CRS/VEH/No AS, P<0.001) and in CRS rats, although in a less accentuated fashion, regardless the treatment (+24% vs. No CRS/VEH/No AS, P<0.05; +23% vs. No CRS/VEH/No AS, P<0.05 of CRS/VEH/AS and CRS/LUR/AS groups, respectively). However, when we compared the increase of Srxn1 of acutely stressed animals to the proper counterpart, the data reach significance in not treated sham animals (+37% vs. No CRS/VEH/No AS, P<0.001), in treated not stressed animals (+28% vs. No

CRS/LUR/No AS, P<0.01) but not in CRS rats that received the vehicle. It's interesting to note that this impairment due to the previous chronic stress exposure was partially normalized by the pharmacological treatment with lurasidone. Indeed, the acute stress-induced up-regulation of Srxn1 was restored in CRS rats treated with the antipsychotic (+24% vs. CRS/LUR/No AS, P<0.05) (Fig.14B).

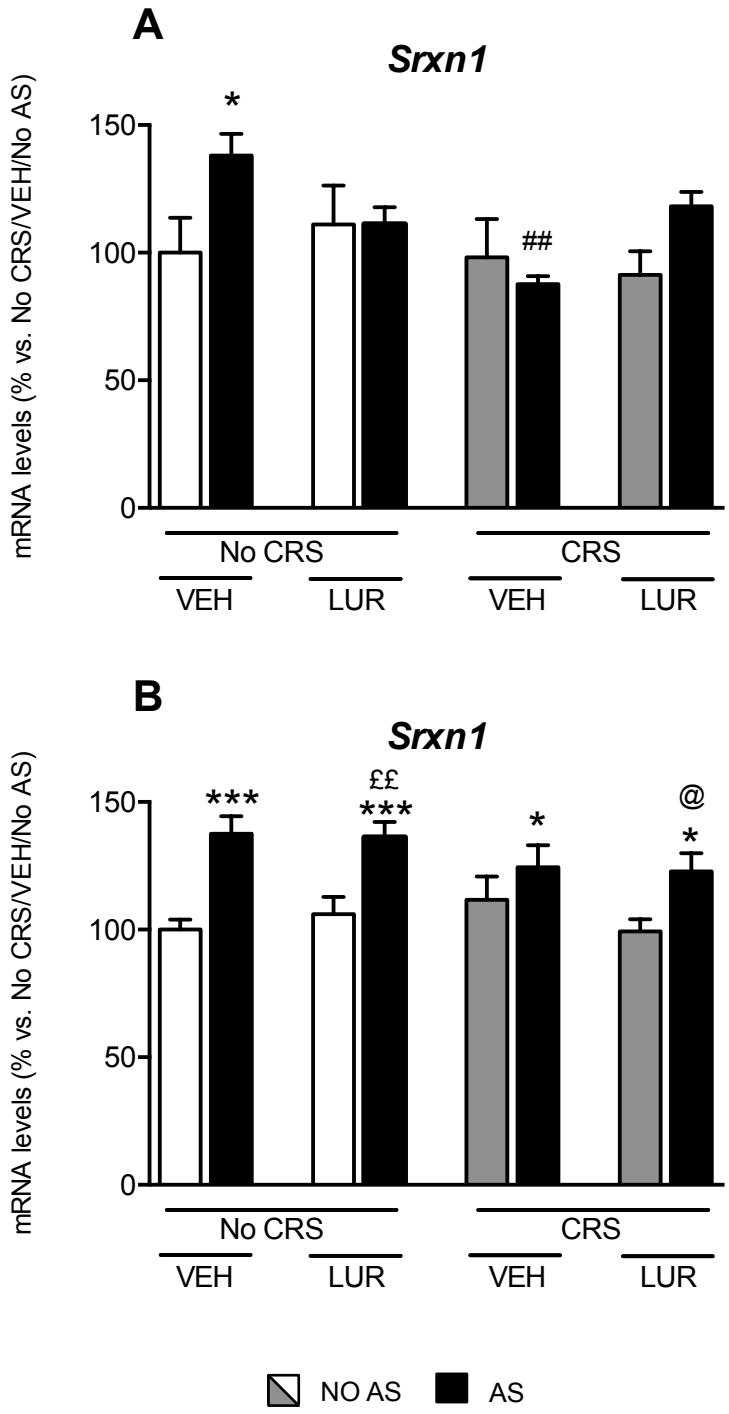

Fig.14 Effect of acute stress exposure on the gene expression of Srxn1 after 3 weeks of washout from CRS and lurasidone treatment

The mRNA levels of Srxn1 were measured in the dorsal (A) and ventral (B) hippocampus of not stressed (No CRS) and stressed (CRS) rats treated with vehicle (VEH) or lurasidone (LUR) and exposed after 3 weeks of washout to acute stress (AS) or not (No AS). The data,

expressed as percentage of No CRS/VEH/No AS animals (set at 100%), are the mean ± SEM of independent determinations. *P<0.05, ***P<0.001 vs. No CRS/VEH/No AS; $^{\#\#}$P<0.01 vs. No CRS/VEH/AS; $^{££}$P<0.01 vs. No CRS/LUR/No AS; $^{@}$P<0.05 vs. CRS/LUR/No AS (Three-way ANOVA with Fisher's LSD).

4.4.2.3 Effect of acute stress exposure on the gene expression of Gpx1, Gpx4 and Mt-1a antioxidant enzymes after 3 weeks of washout from CRS and lurasidone treatment

Given the strong involvement of Srxn1 in the acute antioxidant response to a second challenge, we next examined the gene expression of two further antioxidant enzymes downstream to the transcriptional activity of Nrf2, namely the enzymes cytosolic glutathione peroxidase 1 (Gpx1) and phospholipid-hydroperoxide glutathione peroxidase 4 (Gpx4) and the gene expression of the non-canonical heavy-metal binding antioxidant, metallothionein 1a (Mt-1a) (Fig.15).

Regarding Gpx1 mRNA levels, the Three-way ANOVA analysis in the dorsal hippocampus revealed a significant effect of CRS ($F_{1,59}$=4.440, P=0.0394), a significant CRS x LUR interaction ($F_{1,59}$=19, P<0.0001) and significant CRS x LUR x AS interaction ($F_{1,59}$=7.224, P=0.0093). As a result, we observed a significant reduction of Gpx1 in not treated CRS rats, not exposed (-35% vs. No CRS/VEH/No As, P<0.001) or exposed to acute stress (-26% vs. No CRS/VEH/No As, P<0.01). Interestingly, lurasidone restored Gpx1 levels in CRS animals, resulting in a significant increase compared to CRS/VEH/No AS animals (+52% vs. CRS/VEH/No AS, P<0.001) (Fig.15A). With respect to the ventral hippocampus, the Three-way ANOVA analysis revealed a significant effect of CRS ($F_{1,70}$=8.183, P=0.0056) and significant CRS x LUR x AS interaction ($F_{1,70}$=8.799, P=0.0041). More in detail, we observed a

significant reduction of the antioxidant's mRNA levels in CRS animals exposed to the acute challenge (-28% vs. No CRS/VEH/No AS, P<0.05; -31% vs. No CRS/VEH/AS, P<0.01; -27% vs. CRS/VEH/No AS, P<0.05) and in CRS rats treated with lurasidone (-23% vs. No CRS/VEH/No AS, P<0.05; -22% vs. CRS/VEH/No AS, P<0.05) (Fig.15B).

Concerning Gpx4, in the dorsal hippocampus we observed a significant LUR x AS interaction ($F_{1,65}$=5.358, P=0.0238). Specifically, the analysis highlighted a significant increase of Gpx4 following acute stress in not stressed and not treated animals (+31% vs. No CRS/VEH/No AS, P<0.01) that is not present in animals that received lurasidone (-27% vs. No CRS/VEH/AS, P<0.01) (Fig.15C). The analysis of Gpx4 modulation in the ventral hippocampus revealed a significant effect of lurasidone ($F_{1,68}$=8.525, P=0.0047) and of acute stress ($F_{1,68}$=4.234, P=0.0435). Indeed, in rats not exposed to CRS, Gpx4 gene expression was up-regulated by acute stress only in animals that received lurasidone (+26% vs. No CRS/LUR/No AS, P<0.05). On the other hand, lurasidone was able to increase Gpx4 mRNA levels in CRS animals regardless acute stress exposure (+28% and +42% vs. CRS/VEH/No AS, P<0.05 and P<0.01 of CRS/LUR/No AS and CRS/LUR/AS respectively) (Fig.15D).

The modulation of Mt-1a in the dorsal hippocampus partially reflects the profile of Srxn1. Indeed, as shown by the Three-way ANOVA analyses, Mt-1a was strongly influenced by acute stress ($F_{1,66}$=13.80, P=0.0004) and by a CRS x AS interaction effect ($F_{1,66}$=17.08, P=0.0001). Indeed, acute stress induced the expression of Mt-1a in not stressed rats (+56% vs. No CRS/VEH/No AS, P<0.001) and in not stressed rats treated with lurasidone (+44% vs. No CRS/VEH/No AS, P<0.01; +60% vs. No CRS/LUR/No AS, P<0.001). Interestingly, this acute up-regulation was absent in CRS rats (-29% vs. No CRS/VEH/AS, P<0.01) even in animals treated with lurasidone (-29% vs. No CRS/VEH/AS, P<0.01; -

24% vs. No CRS/LUR/AS P<0.05) (Fig.15E). A partially similar profile was observed in the ventral hippocampus. The Three-way analysis depicted an effect of CRS ($F_{1,70}$=11.54, P=0.0011, of AS ($F_{1,70}$=6.272, P=0.0146) and a CRS x LUR x AS interaction ($F_{1,70}$=4.350, P=0.0407). Acute stress increased Mt-1a in naïve animals (+22% vs. No CRS/VEH/No AS, P<0.05) but this effect could not be seen in animals previously exposed to CRS (-37% vs. No CRS/VEH/AS, P<0.001, -23% vs. No CRS/VEH/No AS, P<0.05). In this regard, lurasidone moderately restore the activation of Mt-1a by acute stress in CRS rats (+19% vs. CRS/LUR/No AS, P<0.05; +24% vs. CRS/VEH/AS, P<0.01) (Fig.15F).

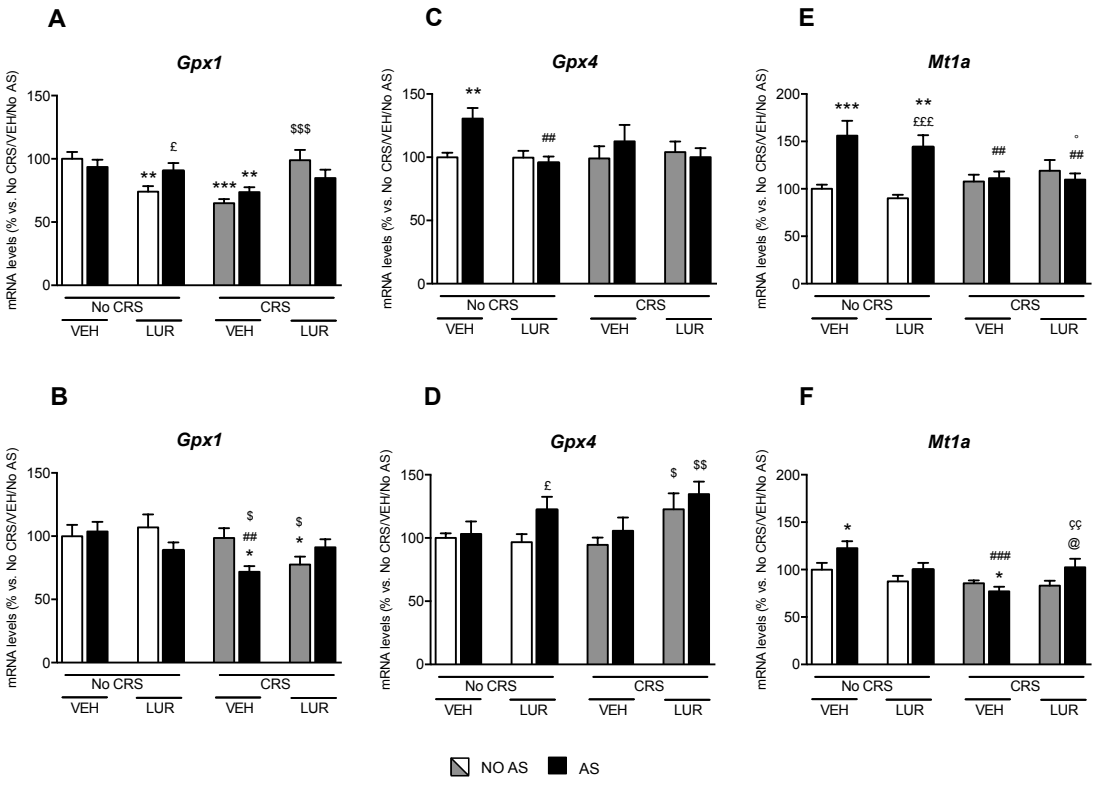

Fig.15 Effect of acute stress exposure on the gene expression of Gpx1, Gpx4 and Mt-1a after 3 weeks of washout from CRS and lurasidone treatment

The mRNA levels of Gpx1, Gpx4 and Mt-1a were measured in the dorsal (A, C, E) and ventral (B, D, F) hippocampus of not stressed (No CRS) and stressed (CRS) rats treated with vehicle (VEH) or lurasidone (LUR) and exposed after 3 weeks of washout to acute stress (AS) or not (No AS). The data, expressed as percentage of No CRS/VEH/No AS animals (set at 100%), are the mean ± SEM of independent determinations. *P<0.05, *P<0.01, ***P<0.001 vs. No CRS/VEH/No AS; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. No CRS/VEH/AS; $^{£}$P<0.05, $^{££}$P<0.01, $^{£££}$P<0.01 vs. No CRS/LUR/No AS; $^{\circ}$P<0.05 vs. No CRS/LUR/AS; $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001 vs. CRS/VEH/No AS; $^{@}$P<0.05 vs. CRS/LUR/No AS, ^{çç}P<0.01 vs. CRS/VEH/AS (Three-way ANOVA with Fisher's LSD).

4.4.3 Discussion

Despite the majority of depressed patients may achieve remission following successful pharmacological treatment, there is a high percentage who experience relapses and reoccurrences, usually as a consequence of environmental adversities. Furthermore, around 30% of the patients did not respond to pharmacological treatments (Vos et al., 2004; Rush et al., 2006), underlying the need to identify novel therapies as well as new pharmacological targets to prevent further relapses.

On these bases, we investigated the recovery effect of a post-stress period and the brain's ability in reacting to a subsequent challenge, in order to identify long-lasting stress-induced changes, and to investigate the ability of lurasidone to improve such alterations. We firstly demonstrated that the molecular signatures produced by chronic restraint stress on redox regulators disappeared after 3 stress-free weeks. Indeed, after recovery, the alterations in the expression of Nox2 and Nrf2 that we observed as a result of chronic mild stress exposure (Rossetti et al., 2018) were no longer detectable in the rat hippocampus. Further, except for Gpx1 in the dorsal hippocampus, also the analyses on other antioxidants did not highlight a long-lasting modulation attributable to CRS. Accordingly, different studies demonstrated that the hippocampus is able to spontaneously "recover" after chronic stress (Ortiz et al., 2014;Ortiz et al., 2018), supporting the adaptive properties of this brain region in re-establishing homeostasis.

Nevertheless, the lack of effects observed after washout cannot exclude the possibility that previous CRS exposure may sensitize oxidative systems to environmental challenges, such as an acute stress. Supporting this theory, we observed that the modulation of Srxn1 and Mt-1a –especially the acute stress-induced up-regulation- was strongly influenced by the previous chronic stress exposure. Given the antioxidant function of Srxn1 and Mt-1a, involved in keeping the balance of the cell's oxidation/reduction, the up-regulation of their mRNA levels

after the acute stress could be seen as a beneficial adaptive reaction of the animal to this specific type of challenge that mimic a sudden difficult situation, a positive effect that is lessened by the previous exposure to a chronic stress. Despite this, we did not observe such a clear modulation of glutathione peroxidases. This could be the result of a different involvement and recruitment of such enzymes in the acute stress response. Indeed, Spiers and colleagues demonstrated that increases of Gpx1 and Gpx4 can be observed in the rat hippocampus after 4 hours of immobilization, while weren't detected after restraint paradigm of 2 or 1 hour, as ours (Spiers et al., 2016).
Moreover, we highlighted the ability of a chronic pharmacological treatment with lurasidone in partially restoring the chronic stress-impaired antioxidant up-regulation of Srxn1 and Mt-1a –an effect limited to the ventral hippocampus for Mt-1a- observed physiologically after acute stress.

Our results suggest that chronic stress-induced oxidative damage may represent a key mechanism contributing to the risk of relapse and to the functional impairments that feature stress-related psychiatric disorders. Indeed, chronic stress left a long-lasting signature, still present after 3 weeks of recovery, and impaired the acute challenge-induced trigger of the antioxidant machinery. Additionally, the results reveal the complexity of the dynamic mechanisms set in motion to cope with challenges, especially when the systems are already impaired, adding new information that should be further investigated to promote adaptive responses. Moreover, we provide new insights on the mechanism of action of lurasidone, which can partially restore the redox homeostasis after stress exposure and may ameliorate specific functions that are deteriorated in psychiatric patients.

5. Summary and Conclusions

In conclusion, the results obtained during my PhD add new preclinical data about how a stress exposure can critically influence our upcoming body reactions to different stimuli. By using different experimental stress approaches, i.e. prenatal stress, social defeat and chronic stress, we strengthen the idea that stress can leave a “scar” in the individuals and affect the outcome of conditions that occur thereafter. Moreover, we brought new insights in the mechanisms set in motion by stress, identifying neuroinflammation, altered oxidative balance and impaired neuroplasticity as potential molecular targets underlying the long-term effects of adverse events.

In the first 2 experiments, I took advantage of the prenatal stress paradigm, a model that allows to deepen the role of early life adversities in shaping a disordered brain. The obtained results showed that being affected by stress during gestation can increase the sensitivity to the EAE encephalomyelitis model of multiple sclerosis in adulthood. Moreover, underlying the more severe symptomatology, we identified alterations in the oligodendrocytes maturation in the spinal cord. Indeed, while during the chronic EAE phase not stressed mice displayed a physiological remyelination, prenatally stressed animals had increased level of GPR17, a marker of immature oligodendrocytes, suggesting that prenatal stress could alter the proper myelin repair during the recovery after the disease. Further, we identified alterations in the signaling of the neurotrophin BDNF in EAE mice exposed to stress, suggesting that the neurotrophin could be a target underlying the stress-induced impaired remyelination.

We next investigated the long-term effect of prenatal stress on the responsiveness to a further acute stress in adulthood. More in detail, after exposing prenatally stressed mice to a second challenge, I analyzed the physiological acute responsiveness of inflammatory markers and mediators of the oxidative balance. The analyses revealed

that fetal stress exposure increased pro-inflammatory and decreased anti-inflammatory cytokines gene expression in the hippocampus, and altered the acute stress modulation of mediators of the oxidative balance. Indeed, once exposed to the second challenge, prenatally stressed mice displayed greater and more lasting nuclear translocation of nuclear factor-like 2 (Nrf-2), a master regulator of the anti-oxidant response, compared to non-stressed animals. However, Nrf-2 up-regulation failed to restore and reactivate the hyperoxidized form of peroxiredoxins (Prxs), which levels are sensitive indicators of oxidative damage. These results suggest that the adaptive mechanisms orchestrated after an acute challenge are in some extent influenced by prenatal stress, and that the physiological re-establishment of homeostasis is impaired by the exposure to early-life adversities.

In the third part of the project I investigated the effect of social stress, a critical feature of adolescence, the period marking the transition from childhood to adulthood. Indeed, adolescence is known to be peculiarly sensitive to the environment and psychosocial stress, such as bullying or subordination, is strongly related to an enhanced vulnerability to psychological morbidities later in adulthood (Jaworska and MacQueen, 2015; Lupien et al., 2009; Romeo & McEwen, 2006). During the 6 months that I spent at the laboratory of Brain Injury, Neuroinflammation and Cognitive Function headed by Professor Susanna Rosi at the University of California, San Francisco, I exposed adolescent male mice to the social defeat stress protocol and then to two mild concussions using a recently developed experimental model of traumatic brain injury (TBI), the repetitive closed-head impact model of engineered rotational acceleration (CHIMERA). The results highlighted that stressed mice developed anxiety-like features, regardless the concussions, while stress and brain injury have a reciprocal influence in the NOR test, where only mice that were both stressed and exposed to TBI did not display impairment in the ability to recognized the novel object. In

parallel to these behavioral –hippocampal-dependent- evaluations I investigated the possible involvement of neuroinflammation in the hippocampus, without finding any difference in microglia activation between the experimental conditions.

In the last part of the thesis, I investigated the long-term effects of the chronic restraint (CRS) paradigm, a well-established animal model of stress-related psychiatric diseases, on the redox homeostasis in the adult rat hippocampus. Specifically, I studied if chronic stress can leave a molecular signature that could still be observed after a recovery of 3 weeks and how this “trace” could alter the responsiveness to a second acute challenge. The molecular analyses targeted modulators of the oxidative balance, recognized to have a role in the etiology of mental diseases, and demonstrated that acute stress strongly induced the gene expression of Srxn1 and Mt-1a, two antioxidant enzymes. This beneficial effect carried out to cope with a sudden challenging situation was impaired by the previous exposure to chronic stress. Interestingly, chronic lurasidone treatment partially restored the appropriate acute responsiveness. Our results suggest that chronic stress-induced oxidative damage may have a critical role in the impaired acute responsiveness that features stress-related psychiatric disorders, and give new insights on the therapeutic mechanism of action of lurasidone.

Since the concept of personalized medicine is becoming more and more acknowledged, the need of taking into account previous environmental stressors in managing patients’ therapies is growing in importance. In this context, my results provide new evidence about possible mechanisms by which stress can prone the individual to be differently susceptible to further adverse events.

6. Bibliography

Acosta, C. M., Cortes, C., MacPhee, H. and Namaka, M. P. (2013) 'Exploring the role of nerve growth factor in multiple sclerosis: implications in myelin repair', CNS Neurol Disord Drug Targets, 12(8), 1242-56.

Al-Harbi, K. S. (2012) 'Treatment-resistant depression: therapeutic trends, challenges, and future directions', Patient Prefer Adherence, 6, 369-88.

Alexander, B. T., Dasinger, J. H. and Intapad, S. (2015) 'Fetal programming and cardiovascular pathology', Compr Physiol, 5(2), 997-1025.

An, J. J., Gharami, K., Liao, G. Y., Woo, N. H., Lau, A. G., Vanevski, F., Torre, E. R., Jones, K. R., Feng, Y., Lu, B. and Xu, B. (2008) 'Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons', Cell, 134(1), 175-87.

Anacker, C. and Hen, R. (2017) 'Adult hippocampal neurogenesis and cognitive flexibility - linking memory and mood', Nat Rev Neurosci, 18(6), 335-346.

Assary, E., Vincent, J. P., Keers, R. and Pluess, M. (2018) 'Gene-environment interaction and psychiatric disorders: Review and future directions', Semin Cell Dev Biol, 77, 133-143.

Autry, A. E. and Monteggia, L. M. (2012) 'Brain-derived neurotrophic factor and neuropsychiatric disorders', Pharmacol Rev, 64(2), 238-58.

Baj, G., Leone, E., Chao, M. V. and Tongiorgi, E. (2011) 'Spatial segregation of BDNF transcripts enables BDNF to differentially shape distinct dendritic compartments', Proc Natl Acad Sci U S A, 108(40), 16813-8.

Barker, D. J. (1998) 'In utero programming of chronic disease', Clin Sci (Lond), 95(2), 115-28.

Barton, G. M. (2008) 'A calculated response: control of inflammation by the innate immune system', J Clin Invest, 118(2), 413-20.

Bernstein, C. N. (2017) 'The Brain-Gut Axis and Stress in Inflammatory Bowel Disease', Gastroenterol Clin North Am, 46(4), 839-846.

Blaze, J., Asok, A., Borrelli, K., Tulbert, C., Bollinger, J., Ronca, A. E. and Roth, T. L. (2017) 'Intrauterine exposure to maternal stress alters Bdnf IV DNA methylation and telomere length in the brain of adult rat offspring', Int J Dev Neurosci, 62, 56-62.

Blugeot, A., Rivat, C., Bouvier, E., Molet, J., Mouchard, A., Zeau, B., Bernard, C., Benoliel, J. J. and Becker, C. (2011) 'Vulnerability to depression: from brain neuroplasticity to identification of biomarkers', J Neurosci, 31(36), 12889-99.

Bock, J., Breuer, S., Poeggel, G. and Braun, K. (2017) 'Early life stress induces attention-deficit hyperactivity disorder (ADHD)-like behavioral and brain metabolic dysfunctions: functional imaging of methylphenidate treatment in a novel rodent model', Brain Struct Funct, 222(2), 765-780.

Bodnar, C. N., Roberts, K. N., Higgins, E. K. and Bachstetter, A. D. (2019) 'A Systematic Review of Closed Head Injury Models of Mild

Traumatic Brain Injury in Mice and Rats', J Neurotrauma, 36(11), 1683-1706.

Boersma, G. J., Lee, R. S., Cordner, Z. A., Ewald, E. R., Purcell, R. H., Moghadam, A. A. and Tamashiro, K. L. (2014) 'Prenatal stress decreases Bdnf expression and increases methylation of Bdnf exon IV in rats', Epigenetics, 9(3), 437-47.

Bondar, N. P. and Merkulova, T. I. (2016) 'Brain-derived neurotrophic factor and early-life stress: Multifaceted interplay', J Biosci, 41(4), 751-758.

Borders, A. A., Aly, M., Parks, C. M. and Yonelinas, A. P. (2017) 'The hippocampus is particularly important for building associations across stimulus domains', Neuropsychologia, 99, 335-342.

Bouvier, E., Brouillard, F., Molet, J., Claverie, D., Cabungcal, J. H., Cresto, N., Doligez, N., Rivat, C., Do, K. Q., Bernard, C., Benoliel, J. J. and Becker, C. (2017) 'Nrf2-dependent persistent oxidative stress results in stress-induced vulnerability to depression', Mol Psychiatry, 22(12), 1795.

Bramlett, H. M. and Dietrich, W. D. (2015) 'Long-Term Consequences of Traumatic Brain Injury: Current Status of Potential Mechanisms of Injury and Neurological Outcomes', J Neurotrauma, 32(23), 1834-48.

Briones-Buixassa, L., Milà, R., Mª Aragonès, J., Bufill, E., Olaya, B. and Arrufat, F. X. (2015) 'Stress and multiple sclerosis: A systematic review considering potential moderating and mediating factors and methods of assessing stress', Health Psychol Open, 2(2), 2055102915612271.

Briscoe, L., Lavender, T. and McGowan, L. (2016) 'A concept analysis of women's vulnerability during pregnancy, birth and the postnatal period', J Adv Nurs, 72(10), 2330-45.

Bryant, R. (2011) 'Post-traumatic stress disorder vs traumatic brain injury', Dialogues Clin Neurosci, 13(3), 251-62.

Burcusa, S. L. and Iacono, W. G. (2007) 'Risk for recurrence in depression', Clin Psychol Rev, 27(8), 959-85.

Buwalda, B., Geerdink, M., Vidal, J. and Koolhaas, J. M. (2011) 'Social behavior and social stress in adolescence: a focus on animal models', Neurosci Biobehav Rev, 35(8), 1713-21.

Calabrese, F., Rossetti, A. C., Racagni, G., Gass, P., Riva, M. A. and Molteni, R. (2014) 'Brain-derived neurotrophic factor: a bridge between inflammation and neuroplasticity', Front Cell Neurosci, 8, 430.

Calcia, M. A., Bonsall, D. R., Bloomfield, P. S., Selvaraj, S., Barichello, T. and Howes, O. D. (2016) 'Stress and neuroinflammation: a systematic review of the effects of stress on microglia and the implications for mental illness', Psychopharmacology (Berl), 233(9), 1637-50.

Casaril, A. M., Domingues, M., Bampi, S. R., de Andrade Lourenço, D., Padilha, N. B., Lenardão, E. J., Sonego, M., Seixas, F. K., Collares, T., Nogueira, C. W., Dantzer, R. and Savegnago, L. (2019) 'The selenium-containing compound 3-((4-chlorophenyl)selanyl)-1-methyl-1H-indole reverses depressive-like behavior induced by acute restraint stress in mice: modulation of oxido-nitrosative stress and inflammatory pathway', Psychopharmacology (Berl).

Case, L. K., Del Rio, R., Bonney, E. A., Zachary, J. F., Blankenhorn, E. P., Tung, K. S. and Teuscher, C. (2010) 'The postnatal maternal environment affects autoimmune disease susceptibility in A/J mice', Cell Immunol, 260(2), 119-27.

Casey, B. J., Jones, R. M. and Hare, T. A. (2008) 'The adolescent brain', Ann N Y Acad Sci, 1124, 111-26.

Caspi, A. and Moffitt, T. E. (2006) 'Gene-environment interactions in psychiatry: joining forces with neuroscience', Nat Rev Neurosci, 7(7), 583-90.

Cattaneo, A. and Riva, M. A. (2016) 'Stress-induced mechanisms in mental illness: A role for glucocorticoid signalling', J Steroid Biochem Mol Biol, 160, 169-74.

Chandler, N., Jacobson, S., Esposito, P., Connolly, R. and Theoharides, T. C. (2002) 'Acute stress shortens the time to onset of experimental allergic encephalomyelitis in SJL/J mice', Brain Behav Immun, 16(6), 757-63.

Chandran, A., Iyo, A. H., Jernigan, C. S., Legutko, B., Austin, M. C. and Karolewicz, B. (2013) 'Reduced phosphorylation of the mTOR signaling pathway components in the amygdala of rats exposed to chronic stress', Prog Neuropsychopharmacol Biol Psychiatry, 40, 240-5.

Chao, M. V. and Hempstead, B. L. (1995) 'p75 and Trk: a two-receptor system', Trends Neurosci, 18(7), 321-6.

Chen, H., Huang, Q., Zhang, S., Hu, K., Xiong, W., Xiao, L., Cong, R., Liu, Q. and Wang, Z. (2019) 'The Chinese Herbal Formula PAPZ Ameliorates Behavioral Abnormalities in Depressive Mice', Nutrients, 11(4).

Chen, Y., Wu, H., Wang, S., Koito, H., Li, J., Ye, F., Hoang, J., Escobar, S. S., Gow, A., Arnett, H. A., Trapp, B. D., Karandikar, N. J., Hsieh, J. and Lu, Q. R. (2009) 'The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination', Nat Neurosci, 12(11), 1398-406.

Chiba, S., Numakawa, T., Ninomiya, M., Richards, M. C., Wakabayashi, C. and Kunugi, H. (2012) 'Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex', Prog Neuropsychopharmacol Biol Psychiatry, 39(1), 112-9.

Chowdhury, S., Shepherd, J. D., Okuno, H., Lyford, G., Petralia, R. S., Plath, N., Kuhl, D., Huganir, R. L. and Worley, P. F. (2006) 'Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking', Neuron, 52(3), 445-59.

Cobley, J. N., Fiorello, M. L. and Bailey, D. M. (2018) '13 reasons why the brain is susceptible to oxidative stress', Redox Biol, 15, 490-503.

Coe, C. L. and Lubach, G. R. (2005) 'Prenatal origins of individual variation in behavior and immunity', Neurosci Biobehav Rev, 29(1), 39-49.

Collaborators, G. N. (2019) 'Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016', Lancet Neurol, 18(5), 459-480.

Columba-Cabezas, S., Iaffaldano, G., Chiarotti, F., Alleva, E. and Cirulli, F. (2009) 'Early handling increases susceptibility to experimental autoimmune encephalomyelitis (EAE) in C57BL/6 male mice', J Neuroimmunol, 212(1-2), 10-6.

Conradi, S., Malzahn, U., Paul, F., Quill, S., Harms, L., Then Bergh, F., Ditzenbach, A., Georgi, T., Heuschmann, P. and Rosche, B. (2013) 'Breastfeeding is associated with lower risk for multiple sclerosis', Mult Scler, 19(5), 553-8.

Coppolino, G. T., Marangon, D., Negri, C., Menichetti, G., Fumagalli, M., Gelosa, P., Dimou, L., Furlan, R., Lecca, D. and Abbracchio, M. P. (2018) 'Differential local tissue permissiveness influences the final fate of GPR17-expressing oligodendrocyte precursors in two distinct models of demyelination', Glia, 66(5), 1118-1130.

Cui, Z., Zhong, Z., Yang, Y., Wang, B., Sun, Y., Sun, Q., Yang, G. Y. and Bian, L. (2016) 'Ferrous Iron Induces Nrf2 Expression in Mouse Brain Astrocytes to Prevent Neurotoxicity', J Biochem Mol Toxicol, 30(8), 396-403.

Darnaudéry, M. and Maccari, S. (2008) 'Epigenetic programming of the stress response in male and female rats by prenatal restraint stress', Brain Res Rev, 57(2), 571-85.

Davis, M. T., Holmes, S. E., Pietrzak, R. H. and Esterlis, I. (2017) 'Neurobiology of Chronic Stress-Related Psychiatric Disorders:

Evidence from Molecular Imaging Studies', Chronic Stress (Thousand Oaks), 1.

De Santi, L., Annunziata, P., Sessa, E. and Bramanti, P. (2009) 'Brain-derived neurotrophic factor and TrkB receptor in experimental autoimmune encephalomyelitis and multiple sclerosis', J Neurol Sci, 287(1-2), 17-26.

Deuschle, M., Hendlmeier, F., Witt, S., Rietschel, M., Gilles, M., Sánchez-Guijo, A., Fañanas, L., Hentze, S., Wudy, S. A. and Hellweg, R. (2018) 'Cortisol, cortisone, and BDNF in amniotic fluid in the second trimester of pregnancy: Effect of early life and current maternal stress and socioeconomic status', Dev Psychopathol, 30(3), 971-980.

Dibble, C. C. and Cantley, L. C. (2015) 'Regulation of mTORC1 by PI3K signaling', Trends Cell Biol, 25(9), 545-55.

Dimitrijević, M., Laban, O., von Hoersten, S., Marković, B. M. and Janković, B. D. (1994) 'Neonatal sound stress and development of experimental allergic encephalomyelitis in Lewis and DA rats', Int J Neurosci, 78(1-2), 135-43.

Diz-Chaves, Y., Astiz, M., Bellini, M. J. and Garcia-Segura, L. M. (2013) 'Prenatal stress increases the expression of proinflammatory cytokines and exacerbates the inflammatory response to LPS in the hippocampal formation of adult male mice', Brain Behav Immun, 28, 196-206.

Donat, C. K., Scott, G., Gentleman, S. M. and Sastre, M. (2017) 'Microglial Activation in Traumatic Brain Injury', Front Aging Neurosci, 9, 208.

Entringer, S., Buss, C. and Wadhwa, P. D. (2015) 'Prenatal stress, development, health and disease risk: A psychobiological perspective-2015 Curt Richter Award Paper', Psychoneuroendocrinology, 62, 366-75.

Faa, G., Manchia, M., Pintus, R., Gerosa, C., Marcialis, M. A. and Fanos, V. (2016) 'Fetal programming of neuropsychiatric disorders', Birth Defects Res C Embryo Today, 108(3), 207-223.

Fancy, S. P., Kotter, M. R., Harrington, E. P., Huang, J. K., Zhao, C., Rowitch, D. H. and Franklin, R. J. (2010) 'Overcoming remyelination failure in multiple sclerosis and other myelin disorders', Exp Neurol, 225(1), 18-23.

Fioranelli, M., Bottaccioli, A. G., Bottaccioli, F., Bianchi, M., Rovesti, M. and Roccia, M. G. (2018) 'Stress and Inflammation in Coronary Artery Disease: A Review Psychoneuroendocrineimmunology-Based', Front Immunol, 9, 2031.

Fonzo, G. A., Ramsawh, H. J., Flagan, T. M., Simmons, A. N., Sullivan, S. G., Allard, C. B., Paulus, M. P. and Stein, M. B. (2016) 'Early life stress and the anxious brain: evidence for a neural mechanism linking childhood emotional maltreatment to anxiety in adulthood', Psychol Med, 46(5), 1037-54.

Frasch, M. G., Lobmaier, S. M., Stampalija, T., Desplats, P., Pallarés, M. E., Pastor, V., Brocco, M. A., Wu, H. T., Schulkin, J., Herry, C. L., Seely, A. J. E., Metz, G. A. S., Louzoun, Y. and Antonelli, M. C. (2018) 'Non-invasive biomarkers of fetal brain development reflecting prenatal stress: An integrative multi-scale multi-species perspective on data collection and analysis', Neurosci Biobehav Rev.

Fraunberger, E. A., Scola, G., Laliberté, V. L., Duong, A. and Andreazza, A. C. (2016) 'Redox Modulations, Antioxidants, and Neuropsychiatric Disorders', Oxid Med Cell Longev, 2016, 4729192.

Fuchs, E. and Flügge, G. (2014) 'Adult neuroplasticity: more than 40 years of research', Neural Plast, 2014, 541870.

Fumagalli, M., Bonfanti, E., Daniele, S., Zappelli, E., Lecca, D., Martini, C., Trincavelli, M. L. and Abbracchio, M. P. (2015) 'The ubiquitin ligase Mdm2 controls oligodendrocyte maturation by intertwining mTOR with G protein-coupled receptor kinase 2 in the regulation of GPR17 receptor desensitization', Glia, 63(12), 2327-39.

Fumagalli, M., Lecca, D., Coppolino, G. T., Parravicini, C. and Abbracchio, M. P. (2017) 'Pharmacological Properties and Biological Functions of the GPR17 Receptor, a Potential Target for Neuro-Regenerative Medicine', Adv Exp Med Biol, 1051, 169-192.

Galli, F., Piroddi, M., Annetti, C., Aisa, C., Floridi, E. and Floridi, A. (2005) 'Oxidative stress and reactive oxygen species', Contrib Nephrol, 149, 240-260.

Gao, W., Wang, W., Peng, Y. and Deng, Z. (2019) 'Antidepressive effects of kaempferol mediated by reduction of oxidative stress, proinflammatory cytokines and up-regulation of AKT/β-catenin cascade', Metab Brain Dis, 34(2), 485-494.

Gardener, H., Munger, K. L., Chitnis, T., Michels, K. B., Spiegelman, D. and Ascherio, A. (2009) 'Prenatal and perinatal factors and risk of multiple sclerosis', Epidemiology, 20(4), 611-8.

Giedd, J. N. and Rapoport, J. L. (2010) 'Structural MRI of pediatric brain development: what have we learned and where are we going?', Neuron, 67(5), 728-34.

Golden, S. A., Covington, H. E., Berton, O. and Russo, S. J. (2011) 'A standardized protocol for repeated social defeat stress in mice', Nat Protoc, 6(8), 1183-91.

Gradus, J. L. (2017) 'Prevalence and prognosis of stress disorders: a review of the epidemiologic literature', Clin Epidemiol, 9, 251-260.

Gray, J. D., Milner, T. A. and McEwen, B. S. (2013) 'Dynamic plasticity: the role of glucocorticoids, brain-derived neurotrophic factor and other trophic factors', Neuroscience, 239, 214-27.

Guardiola-Diaz, H. M., Ishii, A. and Bansal, R. (2012) 'Erk1/2 MAPK and mTOR signaling sequentially regulates progression through distinct stages of oligodendrocyte differentiation', Glia, 60(3), 476-86.

Hanisch, U. K. and Kettenmann, H. (2007) 'Microglia: active sensor and versatile effector cells in the normal and pathologic brain', Nat Neurosci, 10(11), 1387-94.

Harbo, H. F., Gold, R. and Tintoré, M. (2013) 'Sex and gender issues in multiple sclerosis', Ther Adv Neurol Disord, 6(4), 237-48.

Harris, M. L., Oldmeadow, C., Hure, A., Luu, J., Loxton, D. and Attia, J. (2017) 'Stress increases the risk of type 2 diabetes onset in women: A 12-year longitudinal study using causal modelling', PLoS One, 12(2), e0172126.

Hassan, W., Noreen, H., Castro-Gomes, V., Mohammadzai, I., da Rocha, J. B. and Landeira-Fernandez, J. (2016) 'Association of Oxidative Stress with Psychiatric Disorders', Curr Pharm Des, 22(20), 2960-74.

Hata, T., Nishikawa, H., Itoh, E. and Funakami, Y. (2001) 'Anxiety-like behavior in elevated plus-maze tests in repeatedly cold-stressed mice', Jpn J Pharmacol, 85(2), 189-96.

Heesen, C., Gold, S. M., Huitinga, I. and Reul, J. M. (2007) 'Stress and hypothalamic-pituitary-adrenal axis function in experimental autoimmune encephalomyelitis and multiple sclerosis - a review', Psychoneuroendocrinology, 32(6), 604-18.

Hohmann, C. F., Odebode, G., Naidu, L. and Koban, M. (2017) 'Early Life Stress Alters Adult Inflammatory Responses in a Mouse Model for Depression', Ann Psychiatry Ment Health, 5(2).

Hollis, F. and Kabbaj, M. (2014) 'Social defeat as an animal model for depression', ILAR J, 55(2), 221-32.

Jaworska, N. and MacQueen, G. (2015) 'Adolescence as a unique developmental period', J Psychiatry Neurosci, 40(5), 291-3.

Joëls, M. and Krugers, H. J. (2007) 'LTP after stress: up or down?', Neural Plast, 2007, 93202.

Joëls, M., Pu, Z., Wiegert, O., Oitzl, M. S. and Krugers, H. J. (2006) 'Learning under stress: how does it work?', Trends Cogn Sci, 10(4), 152-8.

Justice, N. J. (2018) 'The relationship between stress and Alzheimer's disease', Neurobiol Stress, 8, 127-133.

Kelly, R. R., McDonald, L. T., Jensen, N. R., Sidles, S. J. and LaRue, A. C. (2019) 'Impacts of Psychological Stress on Osteoporosis: Clinical Implications and Treatment Interactions', Front Psychiatry, 10, 200.

Kelly, S. J. and Ismail, M. (2015) 'Stress and type 2 diabetes: a review of how stress contributes to the development of type 2 diabetes', Annu Rev Public Health, 36, 441-62.

Kertes, D. A., Bhatt, S. S., Kamin, H. S., Hughes, D. A., Rodney, N. C. and Mulligan, C. J. (2017) 'methylation in mothers and newborns is associated with maternal exposure to war trauma', Clin Epigenetics, 9, 68.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K. R., Rush, A. J., Walters, E. E., Wang, P. S. and Replication, N. C. S. (2003) 'The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R)', JAMA, 289(23), 3095-105.

Kim, E. J., Pellman, B. and Kim, J. J. (2015) 'Stress effects on the hippocampus: a critical review', Learn Mem, 22(9), 411-6.

Kimbler, D. E., Murphy, M. and Dhandapani, K. M. (2011) 'Concussion and the adolescent athlete', J Neurosci Nurs, 43(6), 286-90.

Konturek, P. C., Brzozowski, T. and Konturek, S. J. (2011) 'Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options', J Physiol Pharmacol, 62(6), 591-9.

Krementsov, D. N. and Teuscher, C. (2013) 'Environmental factors acting during development to influence MS risk: insights from animal studies', Mult Scler, 19(13), 1684-9.

Krishnan, V. and Nestler, E. J. (2008) 'The molecular neurobiology of depression', Nature, 455(7215), 894-902.

Kubera, M., Obuchowicz, E., Goehler, L., Brzeszcz, J. and Maes, M. (2011) 'In animal models, psychosocial stress-induced (neuro)inflammation, apoptosis and reduced neurogenesis are associated to the onset of depression', Prog Neuropsychopharmacol Biol Psychiatry, 35(3), 744-59.

Kwon, E. J. and Kim, Y. J. (2017) 'What is fetal programming?: a lifetime health is under the control of in utero health', Obstet Gynecol Sci, 60(6), 506-519.

Laban, O., Marković, B. M., Dimitrijević, M. and Janković, B. D. (1995) 'Maternal deprivation and early weaning modulate experimental allergic encephalomyelitis in the rat', Brain Behav Immun, 9(1), 9-19.

Lau, C., Rogers, J. M., Desai, M. and Ross, M. G. (2011) 'Fetal programming of adult disease: implications for prenatal care', Obstet Gynecol, 117(4), 978-85.

Lee, P. R., Brady, D. and Koenig, J. I. (2003) 'Corticosterone alters N-methyl-D-aspartate receptor subunit mRNA expression before puberty', Brain Res Mol Brain Res, 115(1), 55-62.

Lesage, J., Del-Favero, F., Leonhardt, M., Louvart, H., Maccari, S., Vieau, D. and Darnaudery, M. (2004) 'Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat', J Endocrinol, 181(2), 291-6.

Linker, R. A., Lee, D. H., Demir, S., Wiese, S., Kruse, N., Siglienti, I., Gerhardt, E., Neumann, H., Sendtner, M., Lühder, F. and Gold, R. (2010a) 'Functional role of brain-derived neurotrophic factor in neuroprotective autoimmunity: therapeutic implications in a model of multiple sclerosis', Brain, 133(Pt 8), 2248-63.

Loebrich, S. and Nedivi, E. (2009) 'The function of activity-regulated genes in the nervous system', Physiol Rev, 89(4), 1079-103.

Lu, C., Dong, L., Zhou, H., Li, Q., Huang, G., Bai, S. J. and Liao, L. (2018) 'G-Protein-Coupled Receptor Gpr17 Regulates Oligodendrocyte Differentiation in Response to Lysolecithin-Induced Demyelination', Sci Rep, 8(1), 4502.

Luoni, A., Berry, A., Calabrese, F., Capoccia, S., Bellisario, V., Gass, P., Cirulli, F. and Riva, M. A. (2014a) 'Delayed BDNF alterations in the prefrontal cortex of rats exposed to prenatal stress: preventive effect of lurasidone treatment during adolescence', Eur Neuropsychopharmacol, 24(6), 986-95.

Luoni, A., Berry, A., Raggi, C., Bellisario, V., Cirulli, F. and Riva, M. A. (2016) 'Sex-Specific Effects of Prenatal Stress on Bdnf Expression in Response to an Acute Challenge in Rats: a Role for Gadd45β', Mol Neurobiol, 53(10), 7037-7047.

Luoni, A., Macchi, F., Papp, M., Molteni, R. and Riva, M. A. (2014b) 'Lurasidone exerts antidepressant properties in the chronic mild stress model through the regulation of synaptic and neuroplastic mechanisms in the rat prefrontal cortex', Int J Neuropsychopharmacol, 18(4).

Luoni, A., Richetto, J., Racagni, G. and Molteni, R. (2015) 'The long-term impact of early adversities on psychiatric disorders: focus on neuronal plasticity', Curr Pharm Des, 21(11), 1388-95.

Lupien, S. J., McEwen, B. S., Gunnar, M. R. and Heim, C. (2009) 'Effects of stress throughout the lifespan on the brain, behaviour and cognition', Nat Rev Neurosci, 10(6), 434-45.

Machado-Vieira, R., Baumann, J., Wheeler-Castillo, C., Latov, D., Henter, I. D., Salvadore, G. and Zarate, C. A. (2010) 'The Timing of Antidepressant Effects: A Comparison of Diverse Pharmacological and Somatic Treatments', Pharmaceuticals (Basel), 3(1), 19-41.

Maghzi, A. H., Etemadifar, M., Heshmat-Ghahdarijani, K., Nonahal, S., Minagar, A. and Moradi, V. (2012) 'Cesarean delivery may increase the risk of multiple sclerosis', Mult Scler, 18(4), 468-71.

Majidi-Zolbanin, J., Doosti, M. H., Kosari-Nasab, M. and Salari, A. A. (2015) 'Prenatal maternal immune activation increases anxiety- and depressive-like behaviors in offspring with experimental autoimmune encephalomyelitis', Neuroscience, 294, 69-81.

Makar, T. K., Bever, C. T., Singh, I. S., Royal, W., Sahu, S. N., Sura, T. P., Sultana, S., Sura, K. T., Patel, N., Dhib-Jalbut, S. and Trisler, D. (2009) 'Brain-derived neurotrophic factor gene delivery in an animal model of multiple sclerosis using bone marrow stem cells as a vehicle', J Neuroimmunol, 210(1-2), 40-51.

Makar, T. K., Trisler, D., Sura, K. T., Sultana, S., Patel, N. and Bever, C. T. (2008) 'Brain derived neurotrophic factor treatment reduces inflammation and apoptosis in experimental allergic encephalomyelitis', J Neurol Sci, 270(1-2), 70-6.

Markham, J. A. and Koenig, J. I. (2011) 'Prenatal stress: role in psychotic and depressive diseases', Psychopharmacology (Berl), 214(1), 89-106.

McCormick, C. M. and Green, M. R. (2013) 'From the stressed adolescent to the anxious and depressed adult: investigations in rodent models', Neuroscience, 249, 242-57.

McEwen, B. S. (1998) 'Stress, adaptation, and disease. Allostasis and allostatic load', Ann N Y Acad Sci, 840, 33-44.

McEwen, B. S., Bowles, N. P., Gray, J. D., Hill, M. N., Hunter, R. G., Karatsoreos, I. N. and Nasca, C. (2015) 'Mechanisms of stress in the brain', Nat Neurosci, 18(10), 1353-63.

McKim, D. B., Niraula, A., Tarr, A. J., Wohleb, E. S., Sheridan, J. F. and Godbout, J. P. (2016a) 'Neuroinflammatory Dynamics Underlie Memory Impairments after Repeated Social Defeat', J Neurosci, 36(9), 2590-604.

Medzhitov, R. (2007) 'Recognition of microorganisms and activation of the immune response', Nature, 449(7164), 819-26.

Mirzaei, F., Michels, K. B., Munger, K., O'Reilly, E., Chitnis, T., Forman, M. R., Giovannucci, E., Rosner, B. and Ascherio, A. (2011) 'Gestational vitamin D and the risk of multiple sclerosis in offspring', Ann Neurol, 70(1), 30-40.

Modgil, S., Lahiri, D. K., Sharma, V. L. and Anand, A. (2014) 'Role of early life exposure and environment on neurodegeneration: implications on brain disorders', Transl Neurodegener, 3, 9.

Molteni, R., Calabrese, F., Cattaneo, A., Mancini, M., Gennarelli, M., Racagni, G. and Riva, M. A. (2009) 'Acute stress responsiveness of the neurotrophin BDNF in the rat hippocampus is modulated by chronic treatment with the antidepressant duloxetine', Neuropsychopharmacology, 34(6), 1523-32.

Molteni, R., Rossetti, A. C., Savino, E., Racagni, G. and Calabrese, F. (2016) 'Chronic Mild Stress Modulates Activity-Dependent Transcription of BDNF in Rat Hippocampal Slices', Neural Plast, 2016, 2592319.

Murray, P. S. and Holmes, P. V. (2011) 'An overview of brain-derived neurotrophic factor and implications for excitotoxic vulnerability in the hippocampus', Int J Pept, 2011, 654085.

Namjoshi, D. R., Cheng, W. H., Bashir, A., Wilkinson, A., Stukas, S., Martens, K. M., Whyte, T., Abebe, Z. A., McInnes, K. A., Cripton, P. A. and Wellington, C. L. (2017) 'Defining the biomechanical and biological threshold of murine mild traumatic brain injury using CHIMERA (Closed

Head Impact Model of Engineered Rotational Acceleration)', Exp Neurol, 292, 80-91.

Ng, F., Berk, M., Dean, O. and Bush, A. I. (2008) 'Oxidative stress in psychiatric disorders: evidence base and therapeutic implications', Int J Neuropsychopharmacol, 11(6), 851-76.

Nielsen, N. M., Bager, P., Stenager, E., Pedersen, B. V., Koch-Henriksen, N., Hjalgrim, H. and Frisch, M. (2013) 'Cesarean section and offspring's risk of multiple sclerosis: a Danish nationwide cohort study', Mult Scler, 19(11), 1473-7.

Nolan, A., Hennessy, E., Krukowski, K., Guglielmetti, C., Chaumeil, M. M., Sohal, V. S. and Rosi, S. (2018) 'Repeated Mild Head Injury Leads to Wide-Ranging Deficits in Higher-Order Cognitive Functions Associated with the Prefrontal Cortex', J Neurotrauma, 35(20), 2425-2434.

Numakawa, T., Suzuki, S., Kumamaru, E., Adachi, N., Richards, M. and Kunugi, H. (2010) 'BDNF function and intracellular signaling in neurons', Histol Histopathol, 25(2), 237-58.

Núñez-Iglesias, M. J., Novío, S., Almeida-Dias, A. and Freire-Garabal, M. (2010) 'Inhibitory effects of alprazolam on the development of acute experimental autoimmune encephalomyelitis in stressed rats', Pharmacol Biochem Behav, 97(2), 350-6.

Ortiz, J. B., Anglin, J. M., Daas, E. J., Paode, P. R., Nishimura, K. and Conrad, C. D. (2018) 'BDNF and TrkB Mediate the Improvement from Chronic Stress-induced Spatial Memory Deficits and CA3 Dendritic Retraction', Neuroscience, 388, 330-346.

Ortiz, J. B., Mathewson, C. M., Hoffman, A. N., Hanavan, P. D., Terwilliger, E. F. and Conrad, C. D. (2014) 'Hippocampal brain-derived neurotrophic factor mediates recovery from chronic stress-induced spatial reference memory deficits', Eur J Neurosci, 40(9), 3351-62.

Paré, W. P. and Glavin, G. B. (1986) 'Restraint stress in biomedical research: a review', Neurosci Biobehav Rev, 10(3), 339-70.

Patki, G., Solanki, N., Atrooz, F., Allam, F. and Salim, S. (2013) 'Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress', Brain Res, 1539, 73-86.

Pechtel, P. and Pizzagalli, D. A. (2011) 'Effects of early life stress on cognitive and affective function: an integrated review of human literature', Psychopharmacology (Berl), 214(1), 55-70.

Plath, N., Ohana, O., Dammermann, B., Errington, M. L., Schmitz, D., Gross, C., Mao, X., Engelsberg, A., Mahlke, C., Welzl, H., Kobalz, U., Stawrakakis, A., Fernandez, E., Waltereit, R., Bick-Sander, A., Therstappen, E., Cooke, S. F., Blanquet, V., Wurst, W., Salmen, B., Bösl, M. R., Lipp, H. P., Grant, S. G., Bliss, T. V., Wolfer, D. P. and Kuhl, D. (2006) 'Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories', Neuron, 52(3), 437-44.

Poo, M. M. (2001) 'Neurotrophins as synaptic modulators', Nat Rev Neurosci, 2(1), 24-32.

Ragnedda, G., Leoni, S., Parpinel, M., Casetta, I., Riise, T., Myhr, K. M., Wolfson, C. and Pugliatti, M. (2015) 'Reduced duration of breastfeeding

is associated with a higher risk of multiple sclerosis in both Italian and Norwegian adult males: the EnvIMS study', J Neurol, 262(5), 1271-7.

Ramirez, K., Fornaguera-Trías, J. and Sheridan, J. F. (2017) 'Stress-Induced Microglia Activation and Monocyte Trafficking to the Brain Underlie the Development of Anxiety and Depression', Curr Top Behav Neurosci, 31, 155-172.

Reader, B. F., Jarrett, B. L., McKim, D. B., Wohleb, E. S., Godbout, J. P. and Sheridan, J. F. (2015a) 'Peripheral and central effects of repeated social defeat stress: monocyte trafficking, microglial activation, and anxiety', Neuroscience, 289, 429-42.

Reader, B. F., Jarrett, B. L., McKim, D. B., Wohleb, E. S., Godbout, J. P. and Sheridan, J. F. (2015b) 'Peripheral and central effects of repeated social defeat stress: monocyte trafficking, microglial activation, and anxiety', Neuroscience, 289, 429-42.

Riise, T., Mohr, D. C., Munger, K. L., Rich-Edwards, J. W., Kawachi, I. and Ascherio, A. (2011) 'Stress and the risk of multiple sclerosis', Neurology, 76(22), 1866-71.

Robinson, A. P., Harp, C. T., Noronha, A. and Miller, S. D. (2014) 'The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment', Handb Clin Neurol, 122, 173-89.

Rohleder, N. (2014) 'Stimulation of systemic low-grade inflammation by psychosocial stress', Psychosom Med, 76(3), 181-9.

Romeo, R. D. (2013) 'The Teenage Brain: The Stress Response and the Adolescent Brain', Curr Dir Psychol Sci, 22(2), 140-145.

Rossetti, A. C., Paladini, M. S., Colombo, M., Gruca, P., Lason-Tyburkiewicz, M., Tota-Glowczyk, K., Papp, M., Riva, M. A. and Molteni, R. (2018) 'Chronic Stress Exposure Reduces Parvalbumin Expression in the Rat Hippocampus through an Imbalance of Redox Mechanisms: Restorative Effect of the Antipsychotic Lurasidone', Int J Neuropsychopharmacol, 21(9), 883-893.

Rossetti, A. C., Papp, M., Gruca, P., Paladini, M. S., Racagni, G., Riva, M. A. and Molteni, R. (2016) 'Stress-induced anhedonia is associated with the activation of the inflammatory system in the rat brain: Restorative effect of pharmacological intervention', Pharmacol Res, 103, 1-12.

Rush, A. J., Trivedi, M. H., Wisniewski, S. R., Nierenberg, A. A., Stewart, J. W., Warden, D., Niederehe, G., Thase, M. E., Lavori, P. W., Lebowitz, B. D., McGrath, P. J., Rosenbaum, J. F., Sackeim, H. A., Kupfer, D. J., Luther, J. and Fava, M. (2006) 'Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report', Am J Psychiatry, 163(11), 1905-17.

Salehi, A., Rabiei, Z. and Setorki, M. (2018) 'Effect of gallic acid on chronic restraint stress-induced anxiety and memory loss in male BALB/c mice', Iran J Basic Med Sci, 21(12), 1232-1237.

Salim, S. (2014) 'Oxidative stress and psychological disorders', Curr Neuropharmacol, 12(2), 140-7.

Salleh, M. R. (2008) 'Life event, stress and illness', Malays J Med Sci, 15(4), 9-18.

Sapolsky, R. M., Romero, L. M. and Munck, A. U. (2000) 'How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions', Endocr Rev, 21(1), 55-89.

Sara, J. D., Prasad, M., Eleid, M. F., Zhang, M., Widmer, R. J. and Lerman, A. (2018) 'Association Between Work-Related Stress and Coronary Heart Disease: A Review of Prospective Studies Through the Job Strain, Effort-Reward Balance, and Organizational Justice Models', J Am Heart Assoc, 7(9).

Schiavone, S., Jaquet, V., Trabace, L. and Krause, K. H. (2013) 'Severe life stress and oxidative stress in the brain: from animal models to human pathology', Antioxid Redox Signal, 18(12), 1475-90.

Schmitt, A., Malchow, B., Hasan, A. and Falkai, P. (2014) 'The impact of environmental factors in severe psychiatric disorders', Front Neurosci, 8, 19.

Sharma, S., Powers, A., Bradley, B. and Ressler, K. J. (2016) 'Gene × Environment Determinants of Stress- and Anxiety-Related Disorders', Annu Rev Psychol, 67, 239-61.

Shors, T. J. (2006) 'Stressful experience and learning across the lifespan', Annu Rev Psychol, 57, 55-85.

Smaga, I., Niedzielska, E., Gawlik, M., Moniczewski, A., Krzek, J., Przegaliński, E., Pera, J. and Filip, M. (2015) 'Oxidative stress as an

etiological factor and a potential treatment target of psychiatric disorders. Part 2. Depression, anxiety, schizophrenia and autism', Pharmacol Rep, 67(3), 569-80.

Solati, J., Asiaei, M. and Hoseini, M. H. (2012) 'Using experimental autoimmune encephalomyelitis as a model to study the effect of prenatal stress on fetal programming', Neurol Res, 34(5), 478-83.

Song, C., Halbreich, U., Han, C., Leonard, B. E. and Luo, H. (2009) 'Imbalance between pro- and anti-inflammatory cytokines, and between Th1 and Th2 cytokines in depressed patients: the effect of electroacupuncture or fluoxetine treatment', Pharmacopsychiatry, 42(5), 182-8.

Soriano, F. X., Léveillé, F., Papadia, S., Higgins, L. G., Varley, J., Baxter, P., Hayes, J. D. and Hardingham, G. E. (2008) 'Induction of sulfiredoxin expression and reduction of peroxiredoxin hyperoxidation by the neuroprotective Nrf2 activator 3H-1,2-dithiole-3-thione', J Neurochem, 107(2), 533-43.

Sotiropoulos, I. (2015) 'The neurodegenerative potential of chronic stress: a link between depression and Alzheimer's disease', Adv Exp Med Biol, 822, 221-2.

Spiers, J. G., Chen, H. J., Cuffe, J. S., Sernia, C. and Lavidis, N. A. (2016) 'Acute restraint stress induces rapid changes in central redox status and protective antioxidant genes in rats', Psychoneuroendocrinology, 67, 104-12.

Stein, D. J., Vasconcelos, M. F., Albrechet-Souza, L., Ceresér, K. M. M. and de Almeida, R. M. M. (2017) 'Microglial Over-Activation by Social

Defeat Stress Contributes to Anxiety- and Depressive-Like Behaviors', Front Behav Neurosci, 11, 207.

Sunico, C. R., Sultan, A., Nakamura, T., Dolatabadi, N., Parker, J., Shan, B., Han, X., Yates, J. R., Masliah, E., Ambasudhan, R., Nakanishi, N. and Lipton, S. A. (2016) 'Role of sulfiredoxin as a peroxiredoxin-2 denitrosylase in human iPSC-derived dopaminergic neurons', Proc Natl Acad Sci U S A, 113(47), E7564-E7571.

Teunis, M. A., Heijnen, C. J., Sluyter, F., Bakker, J. M., Van Dam, A. M., Hof, M., Cools, A. R. and Kavelaars, A. (2002) 'Maternal deprivation of rat pups increases clinical symptoms of experimental autoimmune encephalomyelitis at adult age', J Neuroimmunol, 133(1-2), 30-8.

Tongiorgi, E. (2008) 'Activity-dependent expression of brain-derived neurotrophic factor in dendrites: facts and open questions', Neurosci Res, 61(4), 335-46.

Tyler, W. A., Gangoli, N., Gokina, P., Kim, H. A., Covey, M., Levison, S. W. and Wood, T. L. (2009) 'Activation of the mammalian target of rapamycin (mTOR) is essential for oligodendrocyte differentiation', J Neurosci, 29(19), 6367-78.

Tyler, W. A., Jain, M. R., Cifelli, S. E., Li, Q., Ku, L., Feng, Y., Li, H. and Wood, T. L. (2011) 'Proteomic identification of novel targets regulated by the mammalian target of rapamycin pathway during oligodendrocyte differentiation', Glia, 59(11), 1754-69.

Voorhees, J. L., Tarr, A. J., Wohleb, E. S., Godbout, J. P., Mo, X., Sheridan, J. F., Eubank, T. D. and Marsh, C. B. (2013) 'Prolonged restraint stress increases IL-6, reduces IL-10, and causes persistent

depressive-like behavior that is reversed by recombinant IL-10', PLoS One, 8(3), e58488.

Vos, T., Haby, M. M., Barendregt, J. J., Kruijshaar, M., Corry, J. and Andrews, G. (2004) 'The burden of major depression avoidable by longer-term treatment strategies', Arch Gen Psychiatry, 61(11), 1097-103.

Wainstock, T., Anteby, E. Y., Glasser, S., Lerner-Geva, L. and Shoham-Vardi, I. (2014) 'Exposure to life-threatening stressful situations and the risk of preterm birth and low birth weight', Int J Gynaecol Obstet, 125(1), 28-32.

Walhovd, K. B., Krogsrud, S. K., Amlien, I. K., Bartsch, H., Bjørnerud, A., Due-Tønnessen, P., Grydeland, H., Hagler, D. J., Håberg, A. K., Kremen, W. S., Ferschmann, L., Nyberg, L., Panizzon, M. S., Rohani, D. A., Skranes, J., Storsve, A. B., Sølsnes, A. E., Tamnes, C. K., Thompson, W. K., Reuter, C., Dale, A. M. and Fjell, A. M. (2016) 'Neurodevelopmental origins of lifespan changes in brain and cognition', Proc Natl Acad Sci U S A, 113(33), 9357-62.

Wang, F., Pan, F., Shapiro, L. A. and Huang, J. H. (2017) 'Stress Induced Neuroplasticity and Mental Disorders', Neural Plast, 2017, 9634501.

Wang, M., Ramasamy, V. S., Samidurai, M. and Jo, J. (2019) 'Acute restraint stress reverses impaired LTP in the hippocampal CA1 region in mouse models of Alzheimer's disease', Sci Rep, 9(1), 10955.

Wang, Y. L., Han, Q. Q., Gong, W. Q., Pan, D. H., Wang, L. Z., Hu, W., Yang, M., Li, B., Yu, J. and Liu, Q. (2018) 'Microglial activation mediates

chronic mild stress-induced depressive- and anxiety-like behavior in adult rats', J Neuroinflammation, 15(1), 21.

Weber, M. D., Godbout, J. P. and Sheridan, J. F. (2017) 'Repeated Social Defeat, Neuroinflammation, and Behavior: Monocytes Carry the Signal', Neuropsychopharmacology, 42(1), 46-61.

Weinstock, M. (2005) 'The potential influence of maternal stress hormones on development and mental health of the offspring', Brain Behav Immun, 19(4), 296-308.

Weinstock, M. (2017) 'Prenatal stressors in rodents: Effects on behavior', Neurobiol Stress, 6, 3-13.

Winiarski, D. A., Engel, M. L., Karnik, N. S. and Brennan, P. A. (2018) 'Early Life Stress and Childhood Aggression: Mediating and Moderating Effects of Child Callousness and Stress Reactivity', Child Psychiatry Hum Dev, 49(5), 730-739.

Winston, J. H., Li, Q. and Sarna, S. K. (2014) 'Chronic prenatal stress epigenetically modifies spinal cord BDNF expression to induce sex-specific visceral hypersensitivity in offspring', Neurogastroenterol Motil, 26(5), 715-30.

Xia, B., Chen, C., Zhang, H., Xue, W., Tang, J., Tao, W., Wu, R., Ren, L., Wang, W. and Chen, G. (2016) 'Chronic stress prior to pregnancy potentiated long-lasting postpartum depressive-like behavior, regulated by Akt-mTOR signaling in the hippocampus', Sci Rep, 6, 35042.

Yeh, C. M., Huang, C. C. and Hsu, K. S. (2012) 'Prenatal stress alters hippocampal synaptic plasticity in young rat offspring through

preventing the proteolytic conversion of pro-brain-derived neurotrophic factor (BDNF) to mature BDNF', J Physiol, 590(4), 991-1010.

Yoshii, A. and Constantine-Paton, M. (2010) 'Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease', Dev Neurobiol, 70(5), 304-22.

Young, E. E., Vichaya, E. G., Reusser, N. M., Cook, J. L., Steelman, A. J., Welsh, C. J. and Meagher, M. W. (2013) 'Chronic social stress impairs virus specific adaptive immunity during acute Theiler's virus infection', J Neuroimmunol, 254(1-2), 19-27.

Zhang, Q., Wang, X., Bai, X., Xie, Y., Zhang, T., Bo, S. and Chen, X. (2017) 'Resveratrol reversed chronic restraint stress-induced impaired cognitive function in rats', Mol Med Rep, 16(2), 2095-2100.

Zuccato, C. and Cattaneo, E. (2009) 'Brain-derived neurotrophic factor in neurodegenerative diseases', Nat Rev Neurol, 5(6), 311-22.

Zucchi, F. C., Yao, Y., Ward, I. D., Ilnytskyy, Y., Olson, D. M., Benzies, K., Kovalchuk, I., Kovalchuk, O. and Metz, G. A. (2013) 'Maternal stress induces epigenetic signatures of psychiatric and neurological diseases in the offspring', PLoS One, 8(2), e56967.

Ślusarczyk, J., Trojan, E., Głombik, K., Budziszewska, B., Kubera, M., Lasoń, W., Popiołek-Barczyk, K., Mika, J., Wędzony, K. and Basta-Kaim, A. (2015) 'Prenatal stress is a vulnerability factor for altered morphology and biological activity of microglia cells', Front Cell Neurosci, 9, 82.