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Stress exposure as risk factor for psychiatric disorders: from functional characterization to pharmacological intervention

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Abstract

Stress response involves several mechanisms and mediators that allow individuals to adapt to a changing environment. The effects of stress may be adaptive or maladaptive, based on the timing and intensity of exposure as well as the individual vulnerability. Indeed, while acute stress induces the activation of the hypothalamic pituitary adrenal (HPA) axis to ensure the normal homeostasis and mediates adaptive reactions to cope with such challenges, chronic stress exposure has detrimental and long-lasting effects on brain functions. Actually, stress represents a major susceptibility element for psychiatric disorders and stressful life events are among the environmental factors that contribute to the etiology of major depressive disorder (MDD). However, there are differences in individual susceptibility to develop stress-related disorders, with some person displaying vulnerability to stressful event and others showing resistance to the same adversities. Moreover, it has to be considered that exposure to an adverse situation may leave a permanent 'scar' in the individual, which confer enhanced vulnerability for relapse.

In this context, the overall goal of my studies was to characterize the effects of both chronic and acute stressors on adult male rats to better understand the behavioral outcomes of stressful events as well as the molecular changes that may sustain the pathologic phenotype emerging as a consequence of stress exposure. Furthermore, we investigated if the pharmacological treatment with lurasidone, a drug approved by the Food and Drug Administration for the treatment of different psychiatric conditions, may modulate the behavioral and molecular alterations induced by stress exposure. In particular, lurasidone acts, with high affinity, as antagonist of dopamine D_2 receptor, serotonin 5-HT_{2A} and 5-HT_7 receptors, with moderate affinity as antagonist of adrenergic $\alpha_{2A}$and α_{2C} and as partial agonist of the HT_{1A} receptors.

Here, by employing the chronic mild stress (CMS) paradigm to induce a depressive like behavior in rodents, we identified two populations of stressed animals: one was susceptible to stress whereas the other was resilient to stress, in term of anhedonia. Moreover, independently from the vulnerable and resilient phenotype, we found that all stressed rats developed cognitive deficits. Among the mechanisms underlying the link between cognitive impairment and MDD, we found that the cognitive decline associated with stress exposure may be due to alterations in de-novo protein synthesis at synaptic levels.

Interestingly, we highlighted the ability of the antipsychotic lurasidone to normalize not only the anhedonic phenotype, but also the cognitive impairment induced by the CMS paradigm. Furthermore, known that the function of the HPA axis is altered in psychiatric disorders, we provided evidence for the involvement of lurasidone in counteracting the molecular

abnormalities induced by chronic stress on glucocorticoids receptor function and responsiveness. Additionally, considering that recently epigenetics have been well characterized as potential mechanisms by which environmental factors can lead to the development of different psychiatric disorders, we demonstrated that the chronic stress exposure had long lasting consequences on the HPA axis activity, by acting as modulators of the DNA methylation of key players of the glucocorticoid receptor signaling, and that lurasidone was able to counteract such abnormalities, suggesting that some of its long-term effects may also be related to epigenetic mechanisms.

Subsequently, since a high percentage of depressant patients experience relapse after a period of recovery, we assessed differences in molecular responsiveness to a challenging event, in animals that were originally exposed to a chronic stress paradigm and had received the pharmacological treatment. In particular, we highlight the different ability of prefrontal cortex and dorsal hippocampus to cope with the new challenge, in terms of neuroplastic mechanisms, known to be involved in the adaptation of the brain structures to different demand, including environmental challenges.

In addition, in the field of adaptive response to stress, we demonstrated that a single session of acute restraint stress was able to enhance the cognitive performance with a specific temporal profile, by inducing the activation of selected brain players involved in neuroplasticity, including the immediate early genes and the neurotrophic factor Brain Derived Neurotrophic Factor.

These findings support the fundamental impact of stress exposure during adult life, highlighting its critical effect on systems and pathways through which stress can contribute to the development of stress related disorders. Furthermore, the ability of lurasidone to counteract stress-induced abnormalities provide support to the notion that drugs characterized by a multi receptor profile may be effective in counteracting different pathologic alterations and, speculatively, suggest that such compounds may hold a powerful indication for the treatment of different stress-related disorders.

Riassunto

La risposta allo stress nel nostro organismo richiede l'attivazione di meccanismi molecolari e di mediatori che permettono agli individui di adattarsi continuamente ai diversi stimoli esterni a cui sono giornalmente esposti. Gli effetti dell'esposizione a stress possono essere sia adattativi che mal-adattativi, in base alla durata e all'intensità dell'evento stressante: mentre uno stress acuto attiva l'asse ipotalamo-ipofisi surrene (HPA) per garantire una normale omeostasi e per regolare le reazioni adattative necessarie ad affrontare situazioni stressanti, l'esposizione a stress cronico ha effetti dannosi e duraturi sulle funzioni cerebrali. Infatti, lo stress rappresenta uno dei principali fattori di rischio per lo sviluppo di patologie psichiatriche ed è tra i fattori ambientali maggiormente coinvolti nell'eziologia della depressione. Tuttavia, vi è una diversa suscettibilità tra gli individui nello sviluppare disturbi psichiatrici e le alterazioni dovute a prolungati eventi stressanti possono essere fisiologicamente recuperate o possono lasciare dei segni permanenti che son in grado di conferire un aumentato rischio di vulnerabilità ad ulteriori ricadute.

In questo lavoro abbiamo quindi voluto caratterizzare l'impatto dell'esposizione a stress su ratti maschi adulti, al fine di valutare sia gli effetti a livello comportamentale che i meccanismi molecolari coinvolti a livello cerebrale nello sviluppo del fenotipo patologico indotto dallo stress cronico durante la vita adulta. Inoltre, abbiamo valutato come il trattamento farmacologico con l'antipsicotico di seconda generazione a profilo multi-recettoriale lurasidone, approvato dalla Food and Drug Admnistration per il trattamento dei disturbi dell'umore, sia in grado di modulare le alterazioni comportamentali e molecolari causate dalla prolungata esposizione a stress cronico. Il lurasidone è un antagonista dei recettori D_2, $5HT_{2A}$ e $5HT_7$, agonista dei recettori $\alpha_{2A}$and α_{2C} e agonista parziale dei recettori serotoninergici $HT_{1A.}$ Utilizzando il paradigma di chronic mild stress, modello animale di depressione convalidato per indurre un fenotipo depressivo nei roditori, abbiamo identificato due gruppi di animali stressati: uno suscettibile e l'altro resiliente. Inoltre, abbiamo dimostrato che lo sviluppo di deficit cognitivi, sintomi caratteristici dei pazienti depressi, è indipendente dal fenotipo anedonico, dal momento che sono stati osservati in entrambe le sub-popolazioni di ratti stressati. A livello molecolare, tra i meccanismi sottesi allo sviluppo del deterioramento cognitivo, abbiamo dimostrato come questi deficit, indotti dallo stress, siano associati ad alterazioni nei meccanismi di sintesi proteica a livello sinaptico. Abbiamo inoltre avvalorato le proprietà antidepressive del lurasidone come farmaco in grado di curare non solo l'anedonia, ma anche i deficit cognitivi e la sua capacità di agire a livello dell'asse HPA, noto sistema

deregolato nelle patologie psichiatriche, modulando i meccanismi molecolari alterati dallo stress cronico, nella via genomica e non genomica dei recettori dei glucocorticoidi.

Nel contesto delle patologie psichiatriche, recentemente i meccanismi epigenetici son stati ben caratterizzati come potenziali fattori attraverso cui gli stimoli ambientali possono contribuire allo sviluppo dei disturbi connessi allo stress. Su queste basi abbiamo quindi indagato e mostrato come l'esposizione a stress cronico abbia un effetto permanente e duraturo sull'attività dell'asse HPA, in particolare modulando la metilazione del DNA di geni coinvolti nel signaling del recettore dei glucocorticoidi, e come il trattamento con lurasidone sia in grado di normalizzare queste modificazioni epigenetiche.

Successivamente, siccome un'elevata percentuale di pazienti depressi presenta recidive dopo una completa guarigione, abbiamo evidenziato differenze a livello molecolare nella risposta ad un nuovo stress acuto, in seguito a un periodo di washout sia dallo stress che dal trattamento con lurasidone. In particolare, abbiamo mostrato una differente capacità della corteccia prefrontale e dell'ippocampo dorsale di far fronte ad un nuovo stimolo, in termini di fattori neurotrofici, noti per essere coinvolti nell'adattamento delle strutture cerebrali ai diversi stimoli, compresi gli eventi esterni stressanti.

Infine, per valutare la risposta adattativa a stress di breve durata ed intensità, abbiamo dimostrato come una singola sessione di stress acuto da immobilizzazione possa migliorare la performance cognitiva con uno specifico profilo temporale, tramite l'attivazione di alcuni circuiti coinvolti nei meccanismi di neuroplasticità.

In conclusione, i risultati di questo lavoro supportano l'impatto fondamentale dell'esposizione a stress durante la vita adulta, sottolineando che questo abbia un effetto critico su sistemi e meccanismi che possono essere alla base dello sviluppo dei disturbi psichiatrici. Inoltre, la capacità del trattamento farmacologico con lurasidone di contrastare queste modificazioni supporta l'attività multi-recettoriale del farmaco nell'essere efficace nel modulare le differenti alterazioni patologiche correlate alla depressione maggiore e ai disturbi legati allo stress.

Introduction

1.1 What is stress?

The term stress was borrowed from engineering as a measure of the internal forces acting within a deformable body by Hans Selye in the 1930s. He translated this description into biology by defining stress as the results of an organism's failed attempt to respond appropriately to a physical challenge (Selye, 1998). This explanation has been elaborated by including psychological threats by John Mason (Mason, 1959) and in the traditional psychology definition of stress, it occurs when a person perceives the demands of environmental stimuli to be greater than their ability to meet, mitigate or alter those demands (Lazarus and Folkman, 1984;Lazarus et al., 1985).

A subsequent view of stress includes the concepts of the allostasis to describe the adaptive biological processes that preserve "stability through change" (Sterling P, 1988) and of the allostatic load and overload, to refer to the toxic stress effect (Sterling P, 1988;McEwen and Stellar, 1993;McEwen, 1998).

Allostasis is the active process of adapting to daily stressors mediated by cortisol, by the autonomic, metabolic and immune systems, that act together to maintain homeostasis (McEwen, 2006) and are regulated in a balanced way to preserve stability through changes. When allostatic mediators are not turned off or are not produced in a stable manner, they can cause unhealthy changes in brain and body, thus leading to the so-called "allostatic load", that refers to the cumulative effect of multiple stressors, with the dysregulation of the homeostatic network that involves not shutting off the response efficiently, not turning on an adequate response in the first place, or not habituating to the recurrence of the same stressor, thus dampening the allostatic response. The strongest dysfunctions of these mechanisms refer to the "allostatic overload", that denotes the results of the excess stress, accompanied by damaging behaviors, that lead to the pathology (fig. 1).

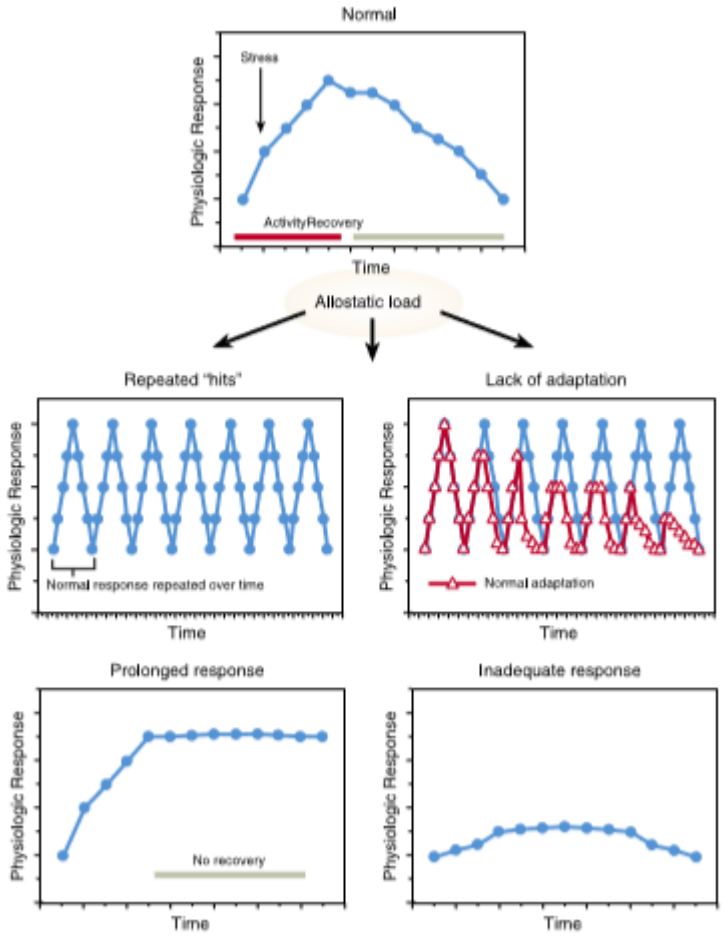

Fig. 1: Illustration of the normal allostatic response, allostatic load and overload. [From (McEwen, 2007)]

These concepts apply not only to the body but also to the brain, the key organ of stress adaptation that provides to ameliorate physiological dysregulation, playing a fundamental role in counteracting or exacerbating allostatic load and overload (McEwen, 2007). Indeed, brain healthy or unhealthy neuronal functions contribute to determining whether the response to challenges is efficient or dysregulated.

Stress physiology, from a practical point of view, can be divided into “positive stress”, that refers to moderate stress response, regulated by stress mediators that promote adaptation during the challenge, being turned on or off when needed; “tolerable stress”, that denotes potentially damaging stress response that can even promote protection against future stressors, with individual internal and external resources that support the system to limit long-term pathophysiological consequences; and “toxic stress” that includes the harmful stress responses occurring under conditions of severe adversity, with the lack of internal or external supplies to cope (Shonkoff et al., 2009), that lead to chronic physiological dysregulation that promotes

diseases (Cohen et al., 2007). Furthermore, these environmental perturbations can be worsened by both genetic and epigenetic mechanism that alters the brain circuity reactivity.

Moreover, the duration is one of the most defining features of a stressor.

"Acute stressors" are intense and short-term exposures, characterized by the occurrence of a specific evoking event. "Daily events", such as deadlines and arguments, are the minor hassles that happen frequently whereas "life events" are time-limited and episodic, and they can have long-term consequences depending on the nature of the event. "Traumatic events" are severe life incidences that can afflict the physical and/or psychological safety of an individual (experiencing violence or abuse, the death of a loved one, natural disaster etc.). Instead, chronic stressors are present for a long period of times and are associated with high negative and low positive daily events (fig.2) (Epel et al., 2018). Enduring stressors may produce chronic states that can lead to the development of mental health problem because of the capability of stress hormones (cortisol in human and corticosterone in rodents) to access the brain and impact affective processing as well as cognitive functioning (Lupien et al., 2007). Chronic stress act as a precipitating factor for many psychiatric conditions, including major depressive disorders (MDD) (Hammen, 2005;Cohen et al., 2007) but it is also linked to a high risk to develop several pathologies, including cardiovascular diseases and infectious diseases (Cohen et al., 2007). In particular, the cost of morbidity associated with mental health conditions exceeds that of any other diseases (Whiteford et al., 2013;DALYs and Collaborators, 2016).

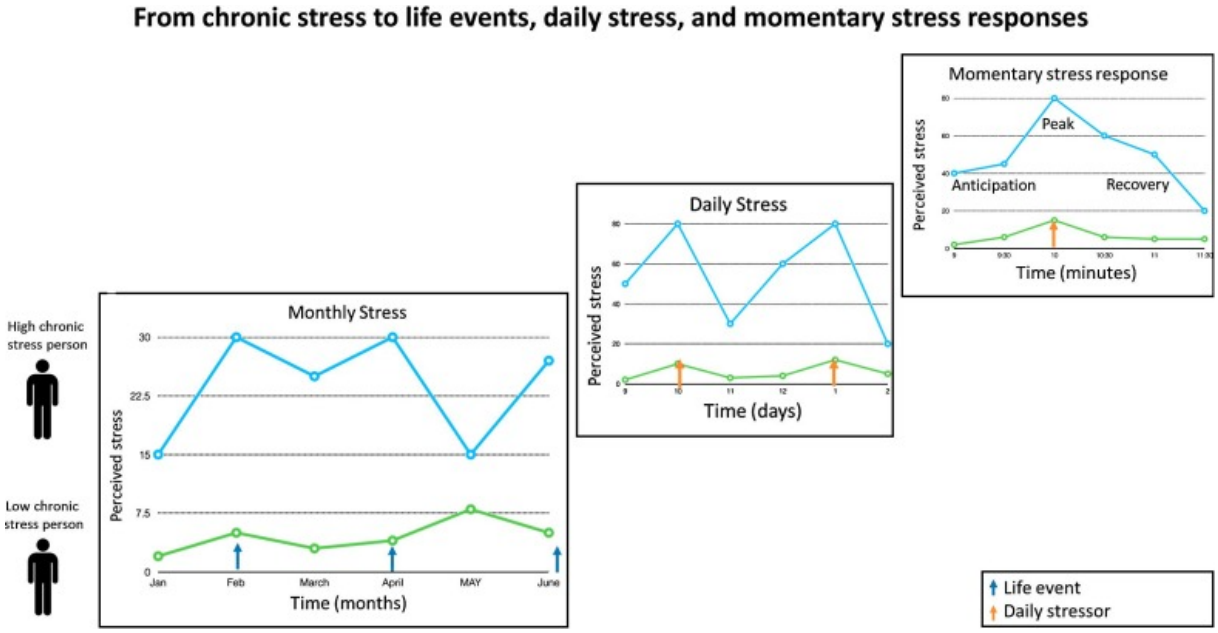

Fig. 2: Time scale of stressors exposure [From (Epel et al., 2018)].

A hallmark of the stress response is the activation of the autonomic nervous system and of the hypothalamic-pituitary-adrenal (HPA) axis, that produce adaptation through "allostasis".
The corticotropin-releasing hormone (CRH) and vasopressin are essential for coordinating the stress response, by acting on the HPA axis, thus regulating the levels of corticosteroid hormones circulating in the blood. In particular, in response to a stressor, neurons in the paraventricular nucleus of the hypothalamus secrete the CRH hormone, 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) from the adrenal glands.
Corticosteroids in the brain operate through mineralocorticoid (MR) and glucocorticoid (GR) receptors, which are co-expressed abundantly in the neurons of limbic structures (Herman et al., 2003). MRs have a high affinity for corticosteroids, so they are mostly occupied even when circulating corticosteroid levels are low (de Kloet et al., 2005). GRs have tenfold lower affinity; consequently, these receptors are only partially bound by corticosteroids under basal conditions and become more occupied as corticosteroid levels increase, for example, after stress.
Both corticosteroids receptors mediate the initiation and the termination of the HPA axis stress response, via the negative feedback, and modulate acquisition processing, storage and retrieval of stressful experiences (Sapolsky et al., 2000). In particular, endogenous glucocorticoids (GCs), in normal physiological circumstances, serve as potent negative regulators of HPA axis activity by binding to their receptors in different tissues, including hypothalamus, pituitary and hippocampus. However, sustained elevations of GCs may disrupt the negative feedback control of the axis, with an excessive activation of the HPA system.
GR and MR exist in membrane-bound form and nuclear form. Indeed, they mediated non-genomic mechanisms and genomic mechanisms (fig. 3). Via the membrane-associated receptors, GCs can directly stimulate the release of excitatory amino acids and indirectly regulate both glutamate and GABA release, through the cross-talk with the endocannabinoid system (Hill and McEwen, 2010). Moreover, GCs can also translocate GRs to mitochondria, where they regulate mitochondrial oxidation, free radical formation, membrane potential and enhance their calcium buffering (Du et al., 2009).
The genomic mechanism involves both direct interactions with glucocorticoid response element (GRE), mediated by cytoplasmic receptors that move to the nucleus and act as transcription factors, and indirect action via tethering to other transcription factors (Revollo and Cidlowski, 2009).

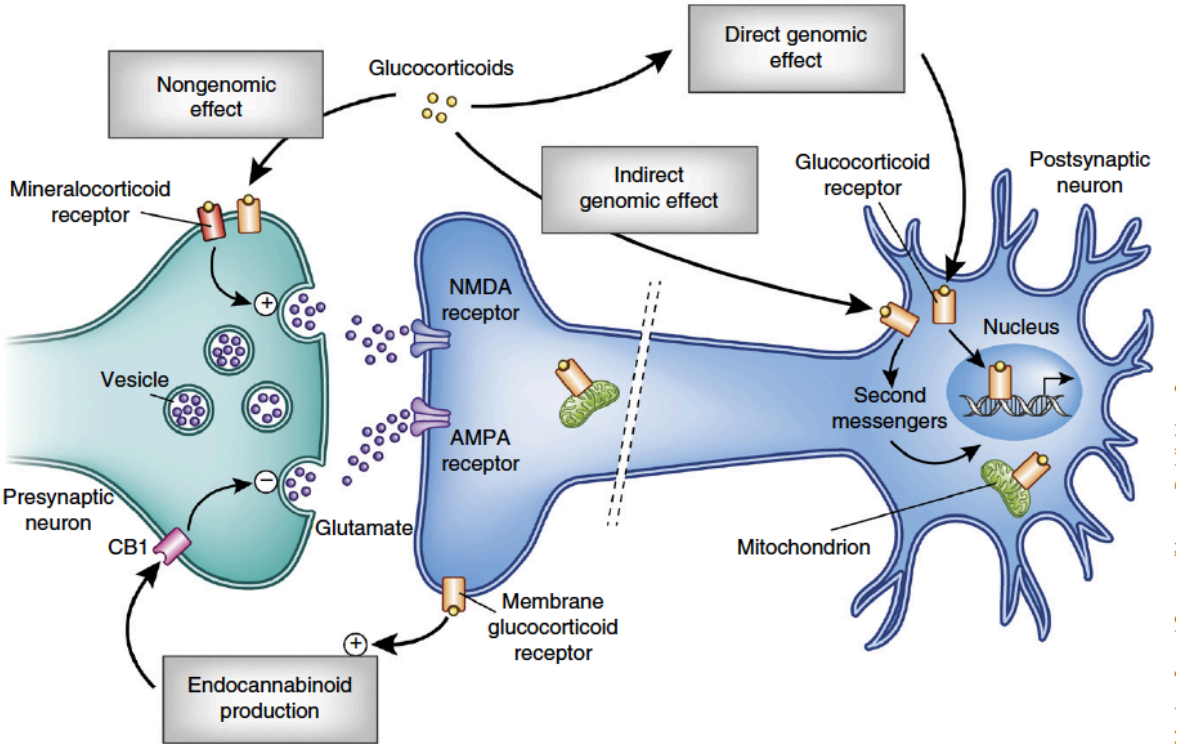

Fig. 3: Genomic and non-genomic mechanisms of glucocorticoids receptor [From (McEwen et al., 2015)].

The glucocorticoid signaling is a complex homeostatic system that cooperates with other network to help the organism to cope with stressful stimuli, in order to adapt to the demand of challenging situation (Chrousos and Kino, 2005;Chrousos, 2009;Nicolaides et al., 2015) in a non-linear interaction. The glucocorticoid effects within the brain are graphically described by the inverted U shape dose-response curve (fig. 4), accordingly with the conceptual work of McEwen that suggest that the stress hormones, essential for survival, can have damaging effects on the brain if they are secreted over longer periods of time (McEwen and Wingfield, 2003). Normal homeostasis is achieved in the central optimal range of the curve, whereas hypofunction or hyperfunction of the HPA axis may have short-term or long-term adverse consequences thus compromising the well-being and/or the performance (Chrousos, 2009;Charmandari et al., 2014;McEwen et al., 2015). Indeed, while exposure to glucocorticoids in term of minutes to hours, such as happens during an acute stress, was associated with low calcium influx but enhanced long-term potentiation (LTP), prolonged periods of stress, associated with high levels of GCs circulating, enhanced cellular calcium exposure and impaired LTP functions (Joels and de Kloet, 1992;Joels et al., 2003a;Joels et al., 2003b;McEwen et al., 2015). An imbalance between MR and GR actions may lead to an altered HPA axis response, thus increasing the vulnerability to affective disorders (De Kloet et al., 1998) and corticosteroid receptor function has been found to be altered in many patients with major depressive disorders (Holsboer, 2000). Polymorphisms of genes encoding for both the receptors have been associated with the

disruption of the HPA axis functioning and depression (van West et al., 2006), whereas in mice a reduction of GR signaling activated the axis ad impaired cognition (Oitzl et al., 2001;Ridder et al., 2005) and the GR deletion increased corticosterone levels and induced depressive-like behavior (Tronche et al., 1999;Boyle et al., 2005).

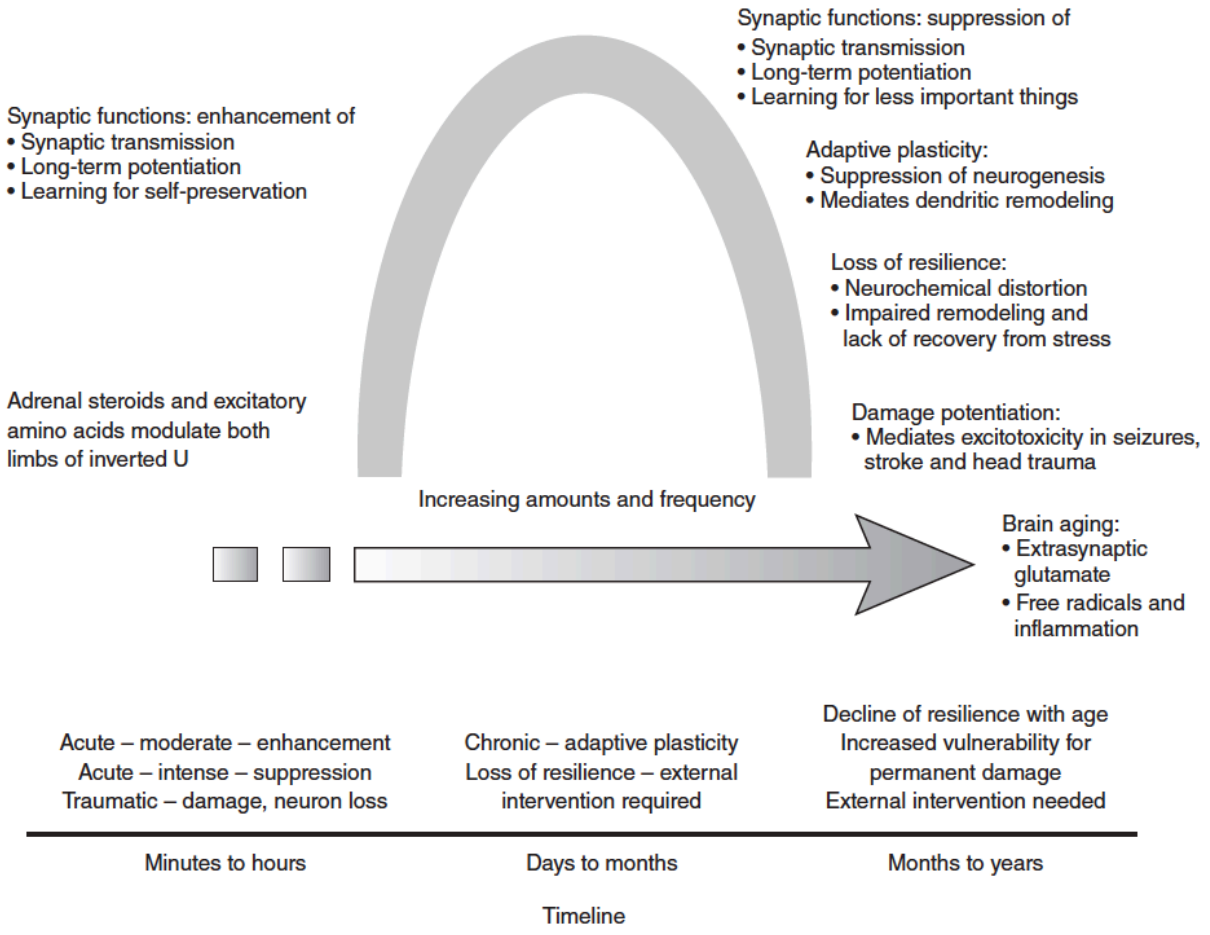

Fig. 4: Inverted U shaped curve of glucocorticoid effects in dose and time [From (McEwen et al., 2015)]

1.2 Stress resilience

Although stress is the major risk factor for psychopathologies, there are differences in the individuals' susceptibility to stress (Russo et al., 2012;Duclot and Kabbaj, 2013;McEwen et al., 2015). Indeed, while some people display a high vulnerability following exposure to stress, others show a resistance to its maladaptive effects (Sandi and Richter-Levin, 2009;Russo et al., 2012). This concept is known as resilience, described by Russo and colleagues as the "capacity of an individual to cope negative social, psychological, and biological consequences of extreme stress that would otherwise compromise their psychological or physical well-being" (Russo et al., 2012). Some reports indicate that resilience in humans represents an active, adaptive process, and not simply the absence of pathological responses that occur in more susceptible individuals (Charney et al., 2004;Feder et al., 2009). The determinants of resilience involve different mediators of the stress response system, including neurotransmitters, neuropeptides, hormones, as well as polymorphisms of mineralocorticoid, glucocorticoids and arginine vasopressin receptors (Walker et al., 2017). Moreover, life events can modulate the mechanisms responsible for the active resilience, including brain neurocircuits and epigenetic processes, underlying why stress resilience is not constant during life but may be modified by external stimuli (McEwen et al., 2016). Different animal studies have investigated the concept of resilience by evaluating the ability to avoid some or all the deleterious behavioral effects of chronic stress and the percent of animals exhibiting resilience varies across several stress paradigms (Russo et al., 2012). Indeed, resilient animals show some behavioral adaptations that appear maladaptive, but they exhibit clear resistance to many others, with respect to the diverse chronic stress procedures to which they are exposed (Russo et al., 2012). Actually, there is increasing evidence that stress resilience arises from active coping strategies, both behavioral and molecular, with some pro-susceptible molecular factors which, if removed, promote stress resilience (Tsankova et al., 2006;Christoffel et al., 2011;Christoffel et al., 2012;Anacker et al., 2016;Anacker et al., 2018). In this field, increasing the knowledge about the neurobiological mechanisms that confer resilience on an individual should improve the treatment strategies to prevent the risk of stress-related disorders.

1.3 Stress and memory

Stress can profoundly influence what we learn by orchestrating the signaling of various hormones, peptides and neurotransmitters (Joels and Baram, 2009). In particular, the stress mediators induce long-term adaptive responses, influencing learning and memory both positively and negatively (de Quervain et al., 2009). The most general view is that stress levels produce an inverted U-shaped dose effect in learning, memory and plasticity (Joels et al., 2006). Indeed, as described above, short periods of stress can potentiate memory formation, whereas more severe or prolonged stressors can have deleterious effects upon broad aspects of cognition, both in human and in rodents (Joels et al., 2006;Shors, 2006;Anacker and Hen, 2017).

Several studies investigated the relationship among acute stress, memory and glucocorticoids using pharmacological and genetic tools, showing how short stressors facilitate good learning and memory performance (Lupien et al., 2002;Joels et al., 2006;Lupien et al., 2009). Human studies supported the procognitive effect produced by acute challenges (Porcelli et al., 2008) and oral administration of hydrocortisone improved working memory performance by enhancing the activity in the prefrontal cortex (PFC) (Henckens et al., 2011). Correlating, rodents' studies established that acute stressors mediated the improvement of memory (Yuen et al., 2009;Yuen et al., 2011). However, other findings showed that acute and uncontrollable stress impaired cognitive functions specifically modulated by the prefrontal cortex (Arnsten, 2009;Arnsten et al., 2015), thus underlying how the duration and the severity of the stressors determine the outcome of the response (Hermans et al., 2014).

Chronic stress-induced changes in learning and memory have crucial implications for understanding stress-related disorders, such as depression (de Quervain et al., 2009). Depression is associated with deficits in cognitive capacity, with difficulties in executive functioning, working memory and processing speed (Chakrabarty, 2016;Ahern and Semkovska, 2017) and some cognitive deficits, as depressive symptoms, are listed in the Diagnostic and Statistical Manual of Mental Disorders (DSM5). When these dysfunctions are present, they can cause considerable difficulties and functional interference in individuals' everyday life. Many studies found that a wide range of antidepressants was able to improve cognitive related-symptoms in MDD (Gallassi et al., 2006;Herrera-Guzman et al., 2010) while others failed (Ferguson et al., 2003;Bastos et al., 2013). Moreover, cognitive impairments are residual symptoms, even when depressive disorders are successfully treated, that cause considerable distress (Gotlib and Joormann, 2010;Parlar et al., 2016) and constitute a serious risk for relapse (Paykel et al., 1995).

The clinical relevance of the effect of stress on learning induced several studies targeting the neuroendocrine mechanisms, that underlie the impact of stress on memory (Schwabe, 2017),

with particular respect to the hippocampus, the key structure for memory (Scoville and Milner, 1957) and to others limbic areas, such as the prefrontal cortex (Lupien et al., 1999). Numerous works indeed reported that exposure to elevated levels of corticosteroids impaired memory tasks dependent on the hippocampus (McEwen and Sapolsky, 1995;Kim and Diamond, 2002) and the direct role of cortisol in cognitive dysfunction was based on findings in patients with Cushing's syndrome, characterized by chronic hypercorticosterolemia (Starkman et al., 1992). Similar, rodent's studies showed deficits in hippocampal dependent spatial learning task due to both stress and corticosterone injections (de Quervain et al., 1998).

Moreover, to provide potential advantages in this field, several studies used animal models exhibiting deficits in one or more of the relevant domains of cognition, to investigate the mechanisms underlying impaired cognitive processes observed in MDD and their dependence on mood pathology (fig. 5) (Darcet et al., 2016).

Cognitive Domains and Functions	Behavioral Paradigms in Rodents
Attention	5-choice serial reaction time task (5-CSRTT)
Executive function - cognitive flexibility - inhibitory learning	Attentional set-shifting task (ASST) Reversal Morris water maze Prepulse inhibition (PPI)
Learning and memory	
Working memory	Delayed alternation Y-maze Delayed alternation T-maze Delayed match-to-sample with odors, subjects Modified MWM, BM, RAWM, RAM
Episodic memory	Novel object recognition test Object location recognition Passive avoidance place Social discrimination procedure
Reference spatial memory	Morris water maze (MWM) Barnes maze (BM) Radial arm water maze (RAWM) Object location recognition/ Object-in-place
Associative memory	Contextual/cued fear conditioning Extinction fear conditioning Passive/active avoidance place

Fig. 5: Behavioural paradigms in rodents used to assess cognitive functions in anxiety/depression models [Adapted from (Darcet et al., 2016)].

1.4 Stress and Major depression

Depressive disorders are among the most widespread forms of psychiatric pathology. According to the World Health Organization, about 350 million people are affected by a depressive disorder (Flint and Kendler, 2014) and by 2020, major depression will be the second-leading cause of disability throughout the world after ischemic heart disease (Murray and Lopez, 1996). MDD is a disorder not limited to adult and elderly population, with a high percentage of patients that experience their first episodes of MDD during childhood and adolescence. In particular, in the early onset of the pathology, individuals typically continue to suffer during adulthood as well. For most people, MDD is a life-long episodic disorder with multiple recurrences with approximately 20%–25% of patients experiencing a chronic, unremitting course (Mueller and Leon, 1996). Functional recovery is critical for patients to remain in remission of MDD and return to productive and fulfilling daily lives (Zimmerman et al., 2006). Moreover, it has been estimated that one third of patients do not achieve remission after well-delivered treatments (Thase, 2010;Greden, 2013). Therefore, recovery of stress-induced changes in neural architecture after stress is not a reversal but a form of neuroplastic adaptation that is impaired in mood disorders (Russo et al., 2012).

Since the 1960s, depression has been diagnosed as "major depression" based on symptomatic criteria set forth in the Diagnostic and Statistical Manual. In particular, the diagnosis is made when a certain number of symptoms (irritability, low-self-esteem, feeling of hopelessness, worthlessness and guilt, decreased ability to concentrate and think, decreased or increased appetite, weight loss or weight gain, insomnia or hypersomnia, low fatigue or increased agitation, decreased interest in pleasurable stimuli, recurrent thought of death and suicide) are reported for longer than a 2 weeks period of time, and when the symptoms disrupt normal, social and occupational functioning. In addition, cognitive impairment is a common symptom of MDD, that often persists even after remission and during recurrent episodes.

Despite the high prevalence of the disease, depressive disorder is complex and heterogeneous, with an etiology still unknown, based upon genetic predisposition and environmental factors that contribute to the development of the pathology. Indeed, even if there is good evidence that episodes of depression often occur in the context of stressful events, stress per se is not sufficient to cause depression. Most people do not become depressed after serious stressful experiences, whereas many develop the pathology. Stressful life events are known to precipitate depressive illness in individuals with certain genetic predispositions (Fava and Kendler, 2000;Caspi et al., 2003) and severe stressful life events seem to be more deleterious for subjects who have a family history of mood disorders. A wide range of environmental events has been

associated with the onset of depression (Kessler, 1997) and a range of difficulties early in life increase the risk of developing the pathology at adulthood (Fava and Kendler, 2000).

Among the environmental factors that can produce long-lasting modification in protein availability and function, epigenetic modifications could explain part of the pathophysiology of depression and of the antidepressant action (Krishnan and Nestler, 2010). Indeed, through epigenetic changes, environmental experiences can modify gene function in the absence of DNA sequence's alterations. Epigenetics has been shown to explain several aspects of depression, including high discordance rates between monozygotic twins, individual differences among inbred rodents, the chronic relapsing nature of the illness and the strikingly greater prevalence of depression in women (Evans et al., 2005).

Family and twin studies have provided strong evidence for the contribution of genetic factors to the risk of depression, with a heritability rate for depression of 37% and data from family studies show a two- to threefold increase in the risk of the illness in the first-degree offspring of patients with depression (Sullivan et al., 2000). Women have been shown to be at greater risk for depressive disorders than men, and the ratio of prevalence rate in women to men has been in the range of 1.5 to 2.5. Different studies have searched for candidate genes involved in the progression of depression since 1978, when the first work on this topic was published (Beckman et al., 1978) and more than 100 candidate genes have been analyzed to identify their possible association with the risk of depression (Shadrina et al., 2018). However, all this study failed to clearly detect the genetic association and underlying mechanism, further supporting that the predisposition to MDD is determined by the coordination of genes action and environmental factors. Altogether, stressors and the genetic predisposition of the individuals create a complex set up that has a relevant impact on the etiology of depression.

Until today, several hypotheses regarding the pathogenesis of depression have been done.

Historically, the first theory was the monoamine hypothesis, proposed by Schildkraut in the 1960s: insufficiency of monoamine neuromediators (serotonin, norepinephrine and dopamine) in defined structures of the central nervous system (CNS) may lead to the development of depression. According to this theory, the synthesis, vesicular transport and receptors of monoamine mediators play an important role in the pathology and the first genetic studies focused on identifying and analyzing polymorphisms in genes associated with serotonin, norepinephrine and dopamine, as well reviewed by Shadrina and colleagues (Shadrina et al., 2018).

The stress-induced theory of depression is based on the statement that hyperactivity of the HPA system, as described above, may be an important mechanism linked with the etiology of the pathology. Indeed, depressed patients frequently show elevated cortisol and corticotropin levels

in plasma (Holsboer and Barden, 1996), increased size of the pituitary and suprarenal glands (Nameroff, 1996) or decreased function of glucocorticoid receptors (Modell et al., 1997). Moreover, excessive activation of the HPA axis is observed in the 50% of depressed people and chronic antidepressants attenuate this activation (Maric and Adzic, 2013).

Another hypothesis proposes a role for neurotrophic factors in the etiology of depression and its treatment (Duman et al., 1997;Altar, 1999), primarily connected with the brain-derived neurotrophic factor (Bdnf). Some studies have reported an association between the Val66Met polymorphism in Bdnf with the depression onset (Schumacher et al., 2005;Ribeiro et al., 2007;Frielingsdorf et al., 2010), even if the effect of this polymorphism has been observed only when associated with other genetic modifications or after exposure to severe stress (Kaufman et al., 2006;Pezawas et al., 2008). Furthermore, it has been found that depressed patients had lower levels of Bdnf in the serum with respect to healthy control subjects and that antidepressant treatment was able to normalize this Bdnf reduction (Shimizu et al., 2003).

In the 1990s the hypothesis of the relationship among the immune system and the CNS in the involvement of neuropathological processes was suggested and in 1999 Maes proposed the inflammatory response system model of depression, that was later extended to the cytokine theory (Maes, 1999). Indeed, depressed patients display increased inflammatory markers in the peripheral blood (Dowlati et al., 2010) and studies on interferon administration showed a high rate of developing depressant symptoms, thus supporting the role of inflammation in depression pathogenesis (Loftis et al., 2004;Asnis and De La Garza, 2006).

Moreover, it has been shown that disturbance of the circadian rhythm system may play a role in the pathology, with mutations of genes encoding for circadian proteins (Gallego and Virshup, 2007;Gouin et al., 2010).

The anatomical basis of depression involves many brain regions, that mediate the diverse symptoms of depression. For example, neocortex and hippocampus mediate cognitive aspects of depression whereas the striatum and amygdala are important in emotional memory, anxiety and motivation (Fava and Kendler, 2000). This is supported by human imaging and anatomic studies in the brain of depressed patients obtained at autopsy, that revealed abnormalities in several subregions including prefrontal and cingulate cortex, hippocampus, striatum, amygdala, thalamus, hypothalamus (Mayberg, 1997;Zhu et al., 1999;Rajkowska, 2000;Drevets, 2001;Liotti and Mayberg, 2001).

1.5 Pharmacological treatments

The treatment of depression was revolutionized about 60 years ago when serendipitously two classes of agents were discovered as antidepressant (Cahn, 2006): the tricyclic antidepressants (TCAs) and the monoamine oxidase inhibitors (MAOIs), which are first generation antidepressants. Laboratory studies revealed that these drugs increased synaptic concentrations of serotonin and norepinephrine and this action was hypothesized to underpin their antidepressant action. They enhance serotoninergic or noradrenergic transmission with several cholinergic side effects, that make them poorly tolerated and dangerous (Cleare et al., 2015). These medications provide a template for a newer class of antidepressants, the second-generation medications: the Selective Serotonin Reuptake Inhibitors (SSRIs) and the Serotonin-Norepinephrine Reuptake Inhibitors (SNRIs). These drugs target selectively the serotoninergic and noradrenergic transporter proteins: in particular, SSRIs and SNRIs block the neuronal serotonin transporter SERT, the neuronal noradrenaline transporter NET, or both, enhancing neurotransmission, presumably by slowing clearance of the transmitter and prolonging its well time in the synapse.

These drugs are the most commonly used medications, which have less toxicity and improved safety compared to first-generation drugs (Millan, 2006;Rush et al., 2006). More recently have been developed drugs that not only block serotonin reuptake but also have additional effects on a variety of serotonin (5-HT) receptor subtypes. For example, vilazodone has partial agonist activity at the 5-HT_{1A} receptor, whereas vortioxetine binds to several other 5-HT receptor subtypes ($5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, $5HT_3$, and 5-HT_7). Additionally, some antidepressant agents do not act through the blockade of norepinephrine and serotonin reuptake. The most widely used is mirtazapine, which blocks α_2-adrenoceptors on norepinephrine cell bodies and terminals, thereby facilitating norepinephrine release, with the antagonism of the 5-HT_{2A} and 5-HT_{2C} receptors, that could also increase norepinephrine and dopamine release in cortical regions (Cleare et al., 2015). A similar antagonist action at 5-HT_{2C} receptors has been suggested to contribute to the antidepressant action of the melatonin agonist agomelatine.

Long-term effects of antidepressant drugs suggest regulatory mechanisms that might contribute to the effectiveness of therapy (Shelton, 2000). However, all of these medications must be given for at least several weeks for their antidepressant actions to become manifest, which means that enhanced serotoninergic or noradrenergic neurotransmission per se is not liable for the clinical actions of these drugs. Despite several decades of research, and many interesting and promising leads, the changes that the drugs induce in the brain, at the bases of their therapeutic actions, remain unclear. An important progress has been done looking for drug-induced plasticity (Nestler et al., 2002) with the assumption that neurobiological adaptive changes, that correlate

in time with the onset of the therapeutic response, could represent a more direct antidepressant target than the initial action of antidepressants to block serotonin and norepinephrine reuptake. Indeed, research has progressed beyond monoamine neurotransmitter receptors to focus on intracellular signaling cascades, gene expression, and protein translation as central for antidepressant drug action and evidence suggests that synaptic plasticity mechanisms are affected by chronic stress, and that antidepressant treatments reverse these effects (Harmer et al., 2017). These findings have defined new potential targets for antidepressant drug discovery, but real challenges remain in translating these to the clinic.

Moreover, second and third-generation antipsychotic appear relevant in the treatment of mood disorders, in particular in the depressive phase of bipolar disorder, of resistant depression and of some anxiety disorders (Jarema, 2007;Javelot, 2016).

1.6 Animal models of depression

Animal models of depression have been generated by employing several methods, including selective breeding, genetic engineering, brain lesions, and environmental manipulations.
In the absence of known highly penetrant genetic causes of depression, much work in animal modeling has relied on the observation that stress and emotional losses are potent risk factors.
In general, for the scientific community there are three main criteria for judging whether a particular disease model is "good enough" to investigate a pathology: the *construct*, the *face* and the *predictive validity*. *Construct validity* refers to the disease relevance of the methods by which a model is constructed: 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 (Chadman et al., 2009); *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 of those treatments in humans.
Among the several animal models of depression employed for their aetiological validity, the main paradigms used to investigate the effect of the environmental factor stress at adulthood are the chronic mild stress (CMS) or "chronic unpredictable" (CUS) stress, the "chronic social defeat stress, the "social isolation" and the "chronic restraint stress" (CRS).
The "chronic mild" or "chronic unpredictable" stress consists of subjecting rodents to repeated physical stressors for weeks or longer periods (Willner, 2005). The protocols allow a combination of a large variety of stressors, that are applied to the animals over a certain period of time, with several number/length of intervals (fig. 6, adapted from (Yin et al., 2016). The model meets the three criteria: at the end of the stress protocol, animals show signs of anhedonia, (face validity), which can be reversed by chronic, but not acute, administration of antidepressant medications (predictive validity) (Nestler and Hyman, 2010). Moreover, 7 weeks of CMS protocol was able to induce not only anhedonia but also cognitive deficit in adult male rats (Calabrese et al., 2017), that were normalized by the prolonged treatment with the antipsychotic lurasidone (Calabrese et al., 2016) (Calabrese et al., manuscript in preparation - chapter 4.2). These behavioral alterations have been connected with molecular abnormalities of neuroplastic mechanisms (Luoni et al., 2015), inflammatory response (Rossetti et al., 2016), circadian rhythms (Calabrese et al., 2016) and local protein synthesis at synaptic level (Calabrese et al., 2017).

Procedure (weeks)	Animal Species	Stressors applied	Stressor intervals
3	Wistar rat	• food or water deprivation • cage tilting • intermittent illumination damp sawdust • grouped housing • low-intensity stroboscopic illumination	Stressors applied individually and continuously with 10–14 intervals
3	SD rat	• food or water deprivation • cage titling and wet cage • continuous overnight illumination • grouped housing • low-intensity stroboscopic illumination	1 or 2 stressors per day and randomized intervals
4	SD rat	• food or water deprivation • continuous overnight illumination • cage titling • grouped housing • damp sawdust • stroboscopic illumination	Stressors applied individually and continuously
4	C57BL/6 mouse	• restraint stress • cage titling • grouped housing • white noise • day-night cycle disturbance	Stressor applied individually for 1 h per day
4	Lister hooded rat	• food or water deprivation • cage tilting • intermittent or overnight illumination • grouped housing • low-intensity stroboscopic illumination • white noise	First 2 weeks: two stressors per day during daylight Last 2 weeks: two stressors per day during night
5	Wistar rat	• food or water deprivation • cage titling • intermittent or stroboscopic illumination • soiled cage • paired housing	Stressor applied individually and continuously with 10–14 intervals
5	SD rat	• grouped housing • cage titling and wet cage • food or water deprivation • stroboscopic illumination • white noise • continuous overnight illumination	1 stressor per day, with or without repetition
6	BALB/c mouse	• grouped housing • noise • damp or remove sawdust • cage changing & tilting • cold water swim • low-intensity stroboscopic illumination • day-night cycle disturbance	1 or 2 stressors per day at different time each day
6	Wistar rat	• food or water deprivation • restraint stress • forced swimming • flashing light • isolation	One stressor per day at different times each day; repeated at random
8	BALB/c mouse	• cage change & tilting • grouped housing • damp or remove sawdust • restraint stress • noise • day-night cycle disturbance	2 stressors per day with randomized combinations and 1–2 h interval
8	Wistar rat	• restraint stress • cage titling • paired housing • nip tail • day-night cycle disturbance	1 stressor per day and each stressor repeated 6–7 times across the procedure
9	Wistar rat	• food or water deprivation • cage tilting • intermittent illumination • soiled cage • grouped housing • low intensity stroboscopic illumination	Stressor applied individually and continuously with 10–14 intervals

Fig. 6: Different protocols of CMS [Adapted from (Yin et al., 2016)].

In the “chronic social defeat stress”, rodents are exposed to repeated bouts of social subordination, that lead to the development of depression-like symptoms, including anhedonia and social withdrawal, which can be reversed by chronic (not acute) antidepressants (Krishnan et al., 2007). This model also induces a metabolic syndrome in mice (Chuang et al., 2010), consistent with homeostatic abnormalities observed in depression. Thus, the social defeat paradigm exhibits feature of construct, face, and predictive validity (Hollis and Kabbaj, 2014). Moreover`, there is recent evidence that prolonged exposure of adult rodents to “social isolation” induces anhedonia, that can be treated effectively with chronic antidepressants (Wallace et al.). In this paradigm rodents are isolated, with differences in the duration and in the age at isolation, on the bases of the protocol employed. Finally, in the “chronic restraint stress” rodents are restrained for at minimum 2 hours per day for 14 to 21 days, protocol that results in depressive behavior in rats. This model, based on continuous and predictable stress, reproduces the repetition of stress that people experience.

Aim

Every day we are exposed to different types of stress that lead to specific biological effects on the bases of their type and duration. The responses to behavioral and physiological stressors are critical for survival under adverse conditions. Dhabhar and McEwen in 1997 gave an integrated definition of stress, which may recapitulate the large amount of information available in the literature: "stress is a constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiological fight or flight systems in the body (stress response) (Dhabhar et al., 1997)". This response can be either normal and adaptive, or abnormal and maladaptive (Selye, 1946). In particular, while exposure to acute stressors induce adaptive reactions that help the organism to cope with the challenges and to adapt efficiently to experiences in daily life, extreme stress conditions both in terms of duration of exposure and intensity of the stressor may lead to a maladaptive outcome, such as the development of psychiatric disorders.

Indeed, stress is the major environmental factor for the etiology of depression (Chrousos and Gold, 1992), and it does it so by inducing stable changes in gene expression, neural circuit function, and behavior, which may be maintained by epigenetic modification. Actually, the interaction with the genetic background seems to be fundamental for the development of the disease (Ohadi et al., 2012), probably explaining the differences response to adverse events observed in humans, with some person displaying susceptibility and others resistance to the maladaptive effects of stress. Indeed, by activating adaptive mechanisms, the brain has the ability to react with the effect due to stressful changing experiences and the capability to cope with negative events exposure, allowing a continuous remodeling throughout the entire life (McEwen et al., 2015). This concept is known as resilience, a process that is at the basis of the differences in the individual susceptibility to stress.

Furthermore, the consequences of chronic stress exposure during adult life may have long-lasting effects or may be recovered, by the activation of dynamic processes to achieve a successful rescue (McDowell et al., 2015). Nevertheless, a high percentage of depressed patients experience relapse after a period of recovery, suggesting that not all the systems impaired by stressful environmental factors are restored, thus representing scars of vulnerability, that in turn can promote the relapse to the pathology.

On these bases, by using a well-established animal model of depression, the main aim of this thesis was to characterize the molecular alterations associated with the development of a pathological phenotype as a consequence of repeated stress exposure as well as to investigate the ability of a pharmacological intervention to counteract or modulate the negative consequences of stress. In particular, we treated rats with the novel antipsychotic lurasidone, approved from the FDA in 2013 for the treatment of mood disorders. In addition, since stress

may also lead to adaptive responses in the brain, based on the timing and on the intensity of the exposure, we evaluated the possible beneficial effects of an acute stress condition.

In particular, with these purposes, the main objectives of this study were to:
- characterize the behavioral phenotype of chronically stressed rats, focusing on the anhedonic phenotype and on the cognitive abilities, to assess the different susceptibility to stress;
- dissect the molecular mechanisms associated with the development of the susceptible and resilient phenotypes;
- investigate the epigenetic mechanisms that may contribute to stress-induced abnormalities;
- identify the molecular alterations that may persist after a recovery period following stress exposure;
- investigate the ability of the pharmacological treatment with the antipsychotic drug lurasidone to correct the behavioral defects induced by stress exposure;
- determine the ability of lurasidone to influence the long-lasting molecular consequences of chronic stress as well as to modulate the response to a new stress later in life;
- characterize the behavioral consequences induced by the exposure to an acute stressor
- evaluate the molecular mechanisms responsible for the adaptive response mediated by the effect of an acute challenge on the cognitive performance.

The molecular mechanism on which we focus on, were analyzed in brain areas of specific interest for stress and related disorders, namely prefrontal cortex and dorsal hippocampus (dHip), because stressful experiences have functionally-relevant effects on these brain area (Kim and Yoon, 1998), that mediate neuroendocrine, autonomic function as well as cognitive and emotional regulation (Kim and Diamond, 2002;McEwen and Gianaros, 2011).

To address these aims, we employed the chronic mild stress paradigm that recapitulates in rodents the features of the illness. Indeed, the CMS paradigm meets the criteria of construct, face and predictive validity, that classify this as a useful animal model to investigate the neurobiological and molecular mechanisms that may be critical in the pathogenesis of major depression.
Our results provided information regarding systems and pathways through which stress can impact brain physiology and contribute to the development of the illness, thus providing new knowledge on the molecular basis of major depression, which may be useful not only for basic science but also for prevention as well as for the treatment of stress-related disorders.

Material and methods

1.7 Animals

Experiments 1 and 2: Male Wistar rats (Charles River, Germany) were brought into the laboratory one month before the start of the experiment. The animals were housed with food and water freely available and were maintained on a 12-h light/dark cycle and in a constant temperature (22 ± 2°C) and humidity (50 ± 5%) conditions. All procedures used in this study have conformed to the rules and principles of the 86/609/EEC Directive and have been approved by the Local Bioethical Committee at the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland.

Experiments 3 and 4: 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 and in a constant temperature (22 ± 2°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.

1.8 Stress procedure

Experiments 1 and 2: Chronic mild stress (CMS). After a period of adaptation to laboratory and housing conditions, the rats were randomly divided into two matched groups. One group (no stress) were housed in separate rooms and had no contact with the stressed animals while the other group of animals was subjected to the chronic mild stress procedure for a period of 7 consecutive weeks. Each week of stress regime consisted of: two periods of food or water deprivation, two periods of 45-degree cage tilt, two periods of intermittent illumination (lights on and off every 2h), two periods of soiled cage (250 ml water in sawdust bedding), one period of paired housing, two periods of low intensity stroboscopic illumination (150 flashes/min), and three periods of no stress. All stressors were 10 - 14 h of duration and were applied individually and continuously, day and night.

Experiment 3: Chronic restraint stress (CRS). After two weeks of adaptation to laboratory and housing conditions, rats were divided into two groups (no stress and CRS). CRS group rats were exposed to an unpredictable chronic restraint stress for 4 weeks. Rats were placed in an air-assessable cylinders (fig. 7) 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 immobile in it. Rats were than left undisturbed for a period of three weeks of recovery (washout). Following the washout period, rats were exposed to one hour of acute restraint stress

(fig. 10).

Experiment 4: *Acute restraint stress.* After two weeks of adaptation to laboratory and housing conditions, rats were exposed to one hour of acute stress, in an air-assessable cylinders (the size of the device was similar to the size of the animal, which made the animal almost immobile in the container).

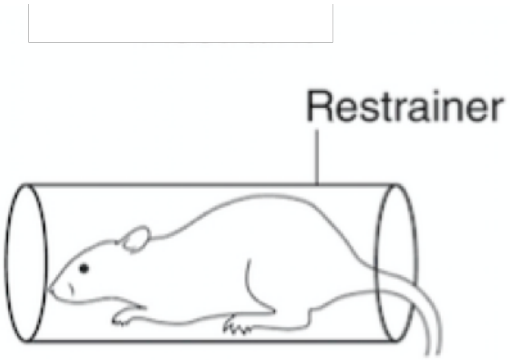

Fig. 7: Schematic representation of the restrainer

1.9 Pharmacological treatment

Experiment 2: Both control and CMS groups, after two weeks of stress protocol, were each divided further into matched subgroups, and for subsequent five weeks they received daily administration of vehicles (1% (w/v) hydroxyethylcellulose, 1 ml/kg, PO) or lurasidone (3 mg/kg, PO). The volume of all injections was 1 ml/kg. The drugs were administered at approx. 10.00 am.

Experiment 3: Starting from the second week of chronic restraint stress protocol, both CRS and control groups were each divided and for three weeks they received once daily administration of vehicles (1% (w/v) hydroxyethylcellulose, 1 ml/kg, PO) or lurasidone (3 mg/kg, PO). The volume of all injections was 1 ml/kg. The drugs were administered in the morning, depending on the random exposure to the restraint stress session.

1.10 Behavioural tests

1.10.1 Sucrose consumption test.

Experiments 1 and 2

To assess the development of the anhedonia, one of the core symptoms of depression, rats were tested at weekly intervals throughout the whole experiments with the sucrose consumption test. In particular, during the period of adaptation in the housing conditions, the animals were trained to consume 1% sucrose solution; training consisted of nine 1h baseline tests, in which sucrose was presented, in the home cage, following 14h food and water deprivation. The sucrose intake was measured by weighing pre-weighed bottles containing the sucrose solution, at the end of the test.

1.10.2 Novel object recognition test

Experiments 1, 2 and 4.

The animals were tested in non-transparent open fields (100 cm in diameter, 35 cm high, with the floor divided into painted 16-cm squares). After 10-min adaptation sessions on two successive days, the animals were allowed to explore two identical objects (white cylinders, 7 cm in diameter, 11 cm high) for the time required to complete 15 s of exploration of both objects (trial session). In the retention trial conducted one hour later (testing session), one of the objects presented previously was replaced by a novel object (black prism, 5 cm wide, 14 cm high). Rats were returned to the open field and the duration of exploration of each object (ie. sitting in close proximity to the objects, sniffing or touching them) was measured during a 5 min test. A NOR index was calculated according to the following formula: time of novel object exploration divided by time of novel plus familiar object exploration, multiplied by 100.

In the experiment 1, at the end of the 7 weeks of chronic mild stress and in the experiment 2, after five weeks of lurasidone administration, all control and stressed animals were randomly divided into two cohorts. One cohort (NOR) was tested in a Novel Object Recognition test and the second cohort (Naive) was left intact (fig. 8 and 9, respectively).

In the experiment 4, non-stressed and acutely stressed rats were divided into subgroups and stressed animals were tested in the NOR 1 hour, 4 hours and 24 hours after the acute challenge (fig. 11).

1.10.3 Locomotor activity test

In the experiment 1, the locomotor activity was monitored in a non-transparent open fields measuring 100 cm in diameter, 35 cm high and with the floor divided into painted 16-cm squares. The number of line crossings was recorded as a measure of locomotor activity.

1.11 Experimental paradigms

The experimental paradigms we designed for our studies are the following:

Experiment 1: Adult male rats were subjected to 7 weeks of chronic mild stress and exposed to the novel object recognition test (NOR) or to the empty arena (Sham)

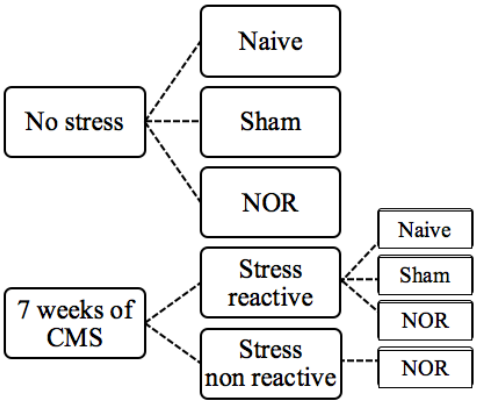

Fig. 8: Schematic representation of the experimental groups of the first paradigm

Experiment 2: Adult male rats were exposed to 7 weeks of chronic mild stressed, treated for 5 weeks with lurasidone (3mg/kg/day) and tested with the novel object recognition test 24 hours after last drug injection

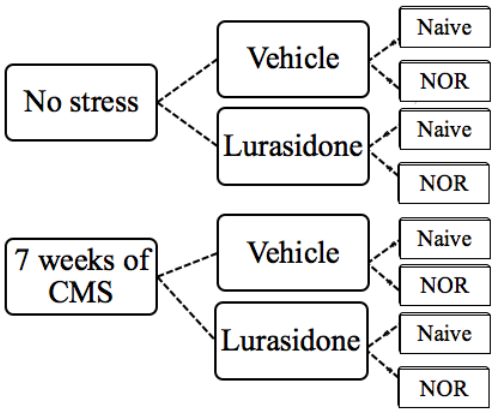

Fig. 9: Schematic representation of the experimental groups of the second paradigm

Experiment 3: Adult male rats were subjected to 4 weeks of chronic restraint stress, treated with lurasidone (3mg/kg/day) for 3 weeks, tested to the novel object recognition and exposed to an acute challenge after three weeks of recover from stress

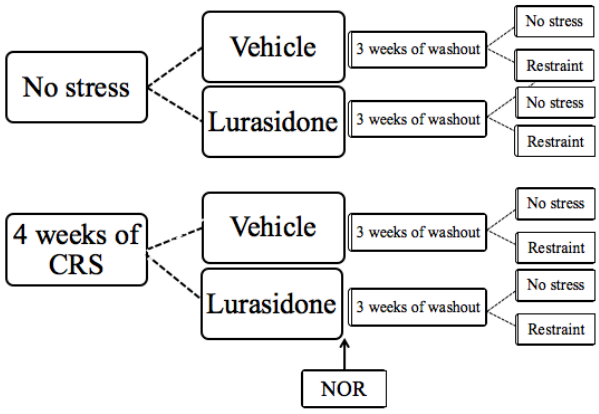

Fig. 10: Schematic representation of the experimental groups of the third paradigm.

Experiment 4: Adult male rats were exposed to 1 hour of restraint stress and tested to the novel object recognition test 1, 4 or 24 hours later

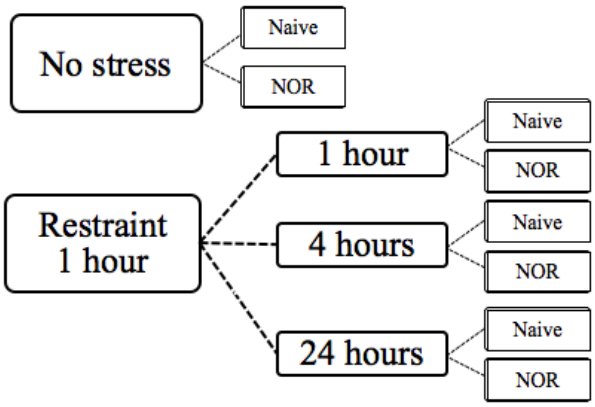

Fig. 11: Schematic representation of the experimental groups of the fourth paradigm.

1.12 Molecular analyses

The molecular analyses were conducted in the prefrontal cortex and in the dorsal hippocampus, dissected from the fresh brain after rat decapitation.
In the experiment 1, rats were killed immediately after the end of the NOR test or to the sham exposure; in the experiment 2, rats were decapitated immediately after the end of the NOR test or 24 hours after the last lurasidone injection. In the experiment 3, rats were killed 1 hour after the acute restraint stress whereas in the experiment 4, 1, 4 or 24 hours after the acute restraint stress or immediately after the end of the NOR test.
Specifically, the prefrontal cortex (defined as Cg1, Cg3 and IL subregions corresponding to the plates 6-10 according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998) was dissected from 2-mm-thick slices, whereas the dorsal hippocampus (plates 25-33 according to the atlas of Paxinos and Watson) was dissected from the whole brain. The brain specimens were then rapidly frozen in dry ice and stored at -80° C for the molecular analyses.

1.12.1 RNA preparation and real time RT-PCR

For gene expression analyses, total RNA was isolated from the different brain regions 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) to assess mRNA levels of several genes (table 1a/b).
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 triplicates as multiplexed reactions with a normalizing internal control (36B4). Thermal cycling was initiated with incubation at 50°C for 10 min (RNA retrotranscription), and then at 95 ºC 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 s to enable the melting process and then for 30 s at 60 °C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression. The primers (f: forward; r: reverse) and probe (p) sequences used were purchased from Eurofins MWG-Operon and are reported in Table 1

a) Gene	Forward primer	Reverse primer	Probe
Arc	GGTGGGTGGCTCTGAAGAAT	ACTCCACCCAGTTCTTCACC	GATCCAGAACCACATGAATGGG
Npas4	TCATTGACCCTGCTGACCAT	AAGCACCAGTTTGTTGCCTG	TGATCGCCTTTTCCGTTGTC
Sgk1	GACTACATTAATGGCGGAGAGC	AGGGAGTGCAGATAACCCAAG	TGCTCGCTTCTACGCAGC
Dusp-1	TGTGCCTGACAGTGCAGAAT	ATCTTTCCGGGAAGCATGGT	ATCCTGTCCTTCCTGTACCT
Fkbp5	GAACCCAATGCTGAGCTTATG	ATGTACTTGCCTCCCTTGAAG	TGTCCATCTCCCAGGATTCTTTGGC
P11	AGAGTGCTCATGGAAAGGGA	AGCTCTGGAAGCCCACTTTT	ATAATGAAAGACCTGGACCAGTGC
Nr3c1	GAAAAGCCATCGTCAAAAGGG	TGGAAGCAGTAGGTAAGGAGA	AGCTTTGTCAGTTGGTAAAACCGTTGC
36B4	TTCCCACTGGCTGAAAAGGT	CGCAGCCGCAAATGC	AAGGCCTTCCTGGCCGATCCATC

b) Gene	Accession number	Assay ID
Gadd45β	BC085337.1	Rn01452530_gI
Nr4a1	BC097313.1	Rn01533237_m1
Cox-1	NC_001665.COX1.0	Rn03296721_s1
Cox-3	NC_001665.COX3.0	Rn03296820_s1

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

1.12.2 Protein extraction and western blot analyses

Western blot analysis was used to investigate GluN1 (phospho Ser896 and total), GluN2B (phospho Ser1303 and total), mTOR (phospho Ser2448 and total), eEF2, (phospho Thr56 and total) OLIGOPHRENIN-1 (OPH-1), BMAL1, ARC, GR and SYNAPSIN I (phospho Ser603 and total) in the subcellular fractions. Tissues were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer containing 0.32 M sucrose, 0.1 mM EGTA, 1 mM HEPES solution in the presence of a complete set of proteases (Roche) and phosphatases (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at 2,500 rpm for 10 min at 4°C to obtain a pellet enriched in nuclear components, which was suspended in a buffer (20 mM HEPES, 0.1 mM dithiothreitol (DTT), 0.1 mM EGTA) with protease and phosphatase inhibitors. The supernatant obtained was further centrifuged at 10,000 g for 15 min at 4°C to obtain the pellet corresponding to the membrane fraction (including both synaptic and mitochondrial contents) which was re-suspended in the same buffer prepared for the nuclear fraction. Total proteins were measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as a calibration standard. Equal amounts of protein were run under reducing conditions on 10% SDS-polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The blots for GR were blocked with BSA in TBS+0,2%sodium azide, while the ones for the other proteins with 10% non-fat dry milk and then were incubated with the primary antibodies summarized in Table 2. Membranes

were then incubated for 1 hour at room temperature with the opportune secondary antibody (see Table 2).

Immunocomplexes were visualized by chemiluminescence using the Western Lightning Plus ECL (Euroclone) and the Chemidoc MP imaging system (Bio-Rad Laboratories). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa.

Protein	Primary antibody	Secondary antibody
phospho GluN1 Ser 896	1:1000 (Santa Cruz),, 4°C, O/N	anti-rabbit, 1:5000, RT, 1h
GluN1	1:1000 (Invitrogen), 4°C, O/N	anti-mouse, 1:3000, RT, 1h
phospho GluN2B Ser1303	1:1000 (Up State), 4°C, O/N	anti-rabbit, 1:2000, RT, 1h
GluN2B	1:1000 (Santa Cruz), 4°C, O/N	anti-goat, 1:2000, RT, 1h
phospho mTOR Ser2448	1:1000 (Cell Signaling), 4°C, O/N	anti-rabbit, 1:1000, RT, 1h
mTOR	1:1000 (Cell Signaling), 4°C, O/N	anti-rabbit, 1:1000, RT, 1h
phospho eEF2 Thr56	1:1000 (Cell Signaling), 4°C, O/N	anti-rabbit 1:1000, RT, 1h
eEF2	1:1000 (Cell Signaling), 4°C, O/N	anti-rabbit 1:1000, RT, 1h
OPHN-1	1:100 (Santa Cruz), 4°C, O/N	anti-mouse 1:1000, RT, 1h
BMAL1	1:5000 (Cell Signaling), 4°C, O/N	anti-rabbit 1:2500, RT, 1h
ARC	1:500 (BD), 4°C, O/N	anti-mouse, 1:1000, RT, 1h
GR	1:500 (ThermoFisher), 4° O/N	1:2000 Anti-rabbit (Cell signaling), 1h RT
phospho SYNAPSIN I Ser603	1:2000 (Upstate), 4° O/N	1:5000 Anti-rabbit (Cell signaling),1h RT
SYNAPSIN Ia/b	1:5000 (Abcam), 4° O/N	1:4000 Anti-goat (Jacksonreasearch), 1h RT
β-Actin	1:10000 (Sigma), 1h RT	1:10000 Anti-mouse (Sigma),1h RT

Table 2: conditions of the antibodies used in western blot analysis (O/N: over/night; RT: room temperature)

1.12.3 DNA extraction, bisulfite treatment and DNA methylation analyses

DNA samples of prefrontal cortex were extracted using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) and stored at −80 °C. An aliquot of 0.5 µg of DNA was treated with sodium-bisulfite with the EZ-96 DNA Methylation-Gold kit (Zymo Research). Both procedures were executed according to the manufacturer's instructions. DNA methylation analysis was carried out using bisulfite-PCR-pyrosequencing. The bisulfite-treated genomic DNA samples were amplified with PCR for the regions of interest. CpG sites within the promoter regions of Nr3c1 and in the glucocorticoid responsive element of Gadd45β and Sgk1 were investigated. Detailed information concerning primer sequences and genomic regions is listed in table 3. Pyrosequencing of the PCR products was carried out using the PyroMark MD Pyrosequencing System (Qiagen) (Bollati et al., 2007). The percentage of 5-methylcytosine (%5mC) was presented as the percentage of cytosine that was methylated divided by the sum of methylated and unmethylated cytosines.

Gene	Chromosome position (RGSC_6.0)	Region	CpG loci	Primers (F), reverse (R) and sequencing (S)
Nr3c1	Chr18:31,728,373-32,704,022	promotor	4	F: biotin-GGGATTTTTAAGAGGTTAGGTAGAG R: ATAACTTTTACTCCCCCACAAATAC S: ACCATAACTCCACCTCATACCC
Gadd45β	Chr7:11,646,283-11,648,338	exon	5	F: Biotin-GTTAAGATAGGAAGGAGGGGATTT R: AAAACAAGAAAACTTAACCAATTT S: ACAATTCACTATCCAAC
Sgk1	Chr1:24,185,451-24,302,309	promotor	2	F: GTATTAGGGTAAGGGTATTGATT R: Biotin-TCATTTCACTTTTTTTTCCAACTA S: TTGTAAGGTTTAAAATTTAT

Table 3. Pyrosequencing assay information (F: forward; R: reverse; S: sequencing).

1.13 Statistical analyses

1.13.1 Behavioral analyses

In the experiments 1 and 4, the results of the behavioral test (sucrose consumption test and/or novel object recognition test) were analyzed with the one-way analysis of variance (ANOVA) followed by Fisher LSD Post Hoc Test (PLSD), whereas in the experiments 2 was used the two-way ANOVA, with stress and pharmacological treatment as independent factors, followed by PLSD. Moreover, in experiments 1 and 2, the analyses of sucrose consumption during the whole stress paradigms were performed with two-way ANOVA with repeated measures. Significance for all tests was assumed for $p<0.05$. Data are presented as means ± standard error (SEM). For graphic clarity, results are presented as means percent of no stress rats.

1.13.2 Molecular analyses

In the experiment 1, the effects of stress (no stress/CMS) and/or the behavioral manipulation (exposure to the empty arena, Sham, or to the NOR test) as independent factors, were analyzed with the two-way ANOVA, followed, when appropriate, by Fisher's protected Least Significant Difference test. In addition, to evaluate the association between the development of cognitive deficits and the alteration of protein expression, Pearson correlation coefficients (r) were evaluated between NOR index of single animals and the corresponding protein levels.
In the experiment 2, the effect of chronic mild tress (no stress/CMS) and of the behavioral manipulations (naïve/NOR), or of the pharmacological treatment (VEH/LUR), as independent factors, were analyzed with the two-way ANOVA, followed, when appropriate, by PLSD.
In the experiment 3, the effect of chronic restraint stress (no stress/CRS), of the treatment (VEH/LUR) as independent factors, were analyzed with the two-way ANOVA, followed, when appropriate, by PLSD. Moreover, the three-way ANOVA with PLSD was used to investigate the effect of chronic restraint stress (no stress/CRS), of the pharmacological treatment (VEH/LUR) and of the acute restraint challenge (no stress/restraint), as independent factors.
In the experiment 4, the effect of the acute stress was analyzed with the one-way ANOVA with PLSD, whereas the two-way ANOVA with PLSD was used to evaluate the effect of both the acute challenge (no stress/ restraint) and the NOR test (naïve/NOR), as independent factors.
Significance for all tests was assumed for $p<0.05$. Data are presented as means ± standard error (SEM). For graphic clarity, results are presented as means percent of no stress rats.

Results and discussion

1.14 Chronic mild stress-induced alterations of local protein synthesis: a role for cognitive impairment

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Depression, a major cause of disability worldwide, is characterized by a complex and heterogeneous symptomatology. With this respect, cognitive deterioration represents a major problem that has a strong impact on patient's function. Indeed, cognitive dysfunctions are characteristic of MDD and are among the persistent symptoms, that can weaken the general performance and cause distress, during both the active phase of the disease and remission. In this context it is not fully clear which are the mechanism underlying the link between cognitive deficits and MDD. Since altered synaptic plasticity has been related with depression and psychiatric disorders, here we focus on de-novo protein synthesis at synaptic levels, to evaluate its possible involvement in the memory maintenance under physiological conditions as well as its potential association with the pathogenesis of mood disorders (Hoeffer and Klann, 2010).
In this study, we employed the chronic mild stress paradigm to induce a depressive like behavior, including cognitive deficits, in adult male rats. At molecular levels, we focus on the neuronal activity and we dissected, at synaptic level, the protein synthesis pathway driven by mammalian target of rapamycin (mTOR) activation, specifically in the dorsal sub region of the hippocampus known to be involved in memory and learning processes. One of the main mechanisms in protein synthesis-dependent memory is the signaling cascades associated with de novo protein synthesis linked with mammalian target of rapamycin complex 1 and the eukaryotic initiation factor 2 (eIF2) (Graber et al., 2013;Trinh and Klann, 2013). Moreover, mTOR activation through NMDA receptors is also involved in peptide elongation, with the involvement of the eukaryotic elongation factor 2 (eEF2) and the translation of specific mRNA (fig. 16B).

1.14.1 Results

1.14.1.1 Behavioral characterization of chronically stressed rats

As shown in figure 12A, one week of chronic mild stress reduced the consumption of 1% sucrose solution, specifically in a sub population of stressed rats, an effect that persisted for the subsequent 6 weeks of CMS. We defined this vulnerable group as “stress reactive”, whereas the rats that did not develop anhedonia were named “stress non-reactive”, since they had, for the whole 7 weeks CMS period, a sucrose intake similar to non-stressed rats.

At the end of the stress protocol, we found that 7 weeks of stress exposure exerted a significant effect on sucrose consumption ($F_{2,40}$=22.40, $p<0.001$) (fig. 12B). Indeed, among stressed rats, about the 80% showed a significant reduction of sucrose intake (-7.6g; $p<0.001$ vs no stress), while the remaining 20% was resilient since they consumed the same amount of sucrose as the control animals (+0,6g; $p>0.05$ vs no stress)

Conversely, when we tested the animals in the novel object recognition, we found that CMS exposure, independently from the anhedonic phenotype produced a significant impairment of the cognitive performance ($F_{2,16}$=9.99, $p<0.01$) (fig. 12C). Indeed, we observed a significant decrease of the NOR index in stress reactive (-37%; $p<0.001$ vs no stress) as well as in stress non-reactive (-30%; $p<0.01$ vs no stress) animals. Notably, stressed rats did not show any defect in the locomotor activity (fig. 12D) ($F_{2,16}$=2.785, $p>0.05$).

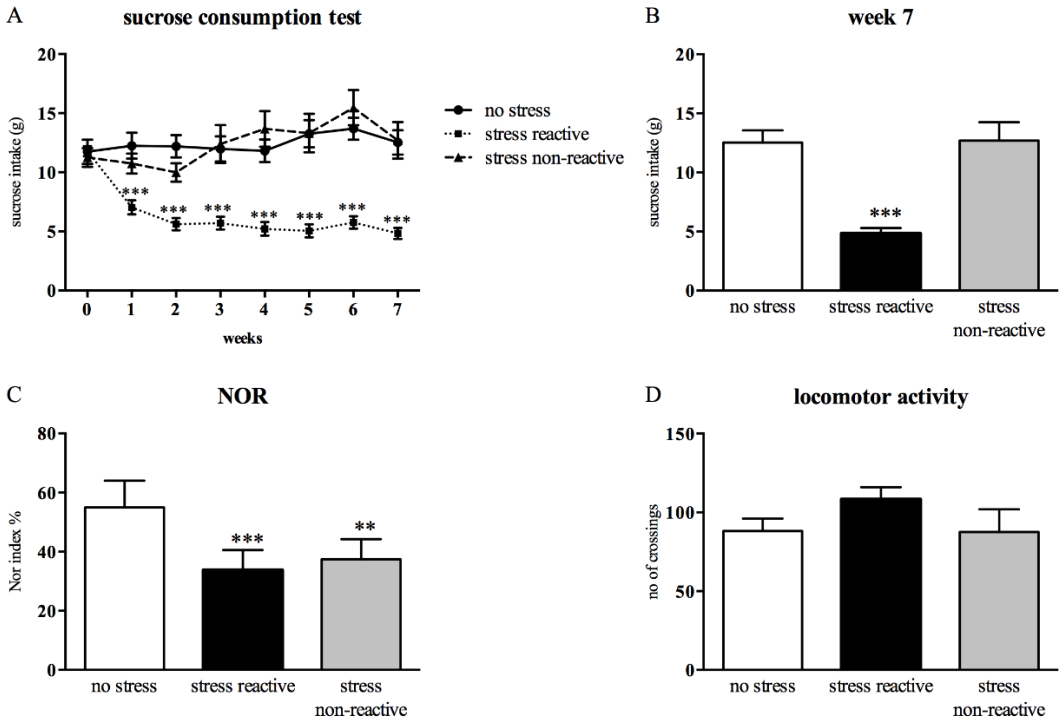

Fig. 12: Behavioural characterization of animals exposed to CMS. Panel A shows the sucrose intake performed every week while panel B reports the results of the test after 7 weeks of CMS. The data are expressed as grams of consumed sucrose. The results of the Novel object recognition test and of the locomotor activity test are summarized in the panel C and D respectively. **p<0.01, ***p<0.001 vs no stress (one-way ANOVA with repeated measures panel A; one-way ANOVA with PLSD panel B-C-D).

1.14.1.2 Modulation of Arc and Npas4 mRNA levels after the Novel object recognition test in the dorsal hippocampus of control and stressed rats

The immediate early genes (IEGs) are markers of neuronal activity. They encode transcription factors that influence neuronal physiology, that in turn regulate late response genes. Even if their role in many biological processes is still unknown, they are related to learning and memory (Gallo et al., 2018), as they are involved in long term memory formation and maintenance in specific neuronal populations (Rosen et al., 1998;Guzowski et al., 1999;Ramirez-Amaya et al., 2005;Minatohara et al., 2015).

Here we focus on *Arc* and *Npas4* gene expression and the molecular analyses were performed in sham animals (who were exposed to the empty arena) as well as in NOR-tested rats, in order to identify specifically the changes produced by the exposure to a novel environment from those linked to the learning process.

Arc and Npas4 genes expression was significantly affected by the behavioral manipulation ($F_{2,35}$=21.588, $p<0.001$; $F_{2,36}$=39.655, $p<0.001$) independently from the stress exposure. Moreover, *Arc* and *Npas4* mRNA levels were significantly increased both in Sham animals (*Arc*: no stress, +393%, $p<0.001$; stress reactive, +192%, $p<0.05$; *Npas4*: no stress, +206%, $p<0.001$; stress reactive: +562%, $p<0.001$) and in those exposed to the test (NOR) (*Arc*: no stress, +296%, $p<0.001$; stress reactive +252%, $p<0.01$; *Npas4*: no stress, +212%, $p<0.001$; stress reactive, +313%, $p<0.001$) (fig. 13A-B).

These changes appear to be due to a non-specific neuronal activation, since we found that the gene expression of this two activity regulated immediate early genes was significantly increased after the NOR test independently from the pre-exposure to the chronic stress procedure. Furthermore, we observed these inductions also in rats exposed to the empty arena (sham groups), suggesting that the neuronal responsiveness is mainly driven by the new experience both in non-stressed and in stressed animals.

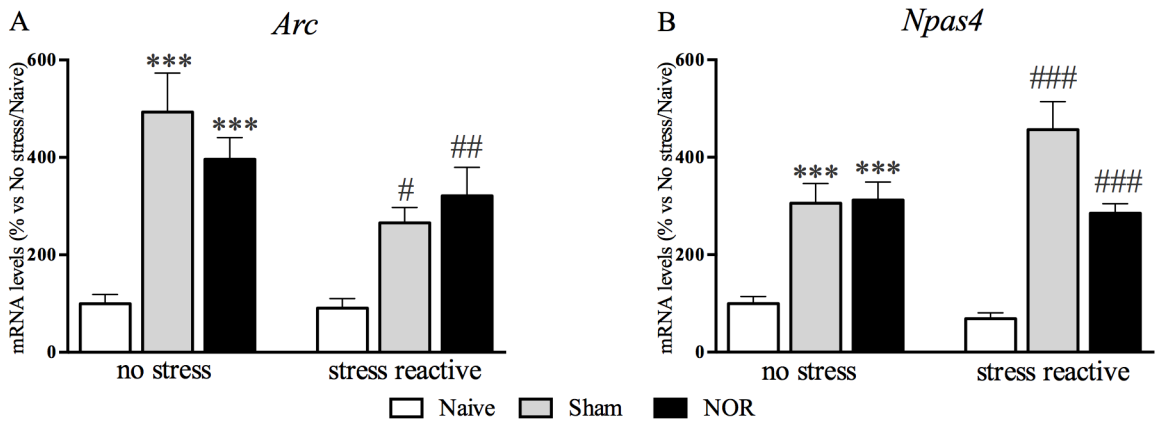

Fig.13: Analysis of Arc and Npas4 mRNA levels in the dorsal hippocampus of chronically stressed rats exposed to the novel object recognition test (NOR). The mRNA levels of *Arc* and *Npas4* were measured in non-stressed and chronically stressed rats exposed to the empty arena (Sham) or to the test (NOR). The data are expressed as a percentage of no stress/Naive (set at 100%). ***p<0.001 vs no stress/Naive, # p<0.05, ## p<0.01, ### p<0.001 vs stress reactive/Naive (two-way ANOVA with PLSD).

1.14.1.3 Modulation of NMDA receptor subunits after the novel object recognition test in the crude synaptosomal fraction of the dorsal hippocampus of control and chronically stressed rats

Among the different pathways implicated in learning and memory formation, the glutamatergic system plays a fundamental role. On this basis, we measured the activated as well as the total form of two NMDA receptor subunits: the mandatory GluN1 and the accessory GluN2B. In the crude synaptosomal fraction, we did not observe any effect on either the phosphorylated (Ser896) (fig. 14A) or the total form of the GluN1 subunit (fig. 14B). On the contrary, for GluN2B activation we found a significant effect of the cognitive test ($F_{2,30}$=21.236, $p<0.001$) of stress ($F_{1,30}$=6,769, $p<0.05$) and a significant stressXtest interaction ($F_{2,30}$=3.503, $p<0.05$) (fig. 14C). Indeed, as confirmed by the post hoc test, phospho-GluN2B Ser1303 levels were significantly increased after the NOR test in no stress animals (+93% $p<0.001$ vs no stress/Naive) but not in those exposed to 7 weeks of CMS (+30% $p>0.05$ vs stress reactive/Naive). Interestingly, we did not observe this effect in animals exposed only to the empty arena (Sham) (no stress: -29% $p>0.05$ vs no stress/Naïve; stress reactive: -33%, $p>0.05$ vs stress reactive/Naive), suggesting that this modulation seems to be specific for the learning processes. Moreover, also the total form of GluN2B (fig. 14D) was significantly affected by the acute manipulation ($F_{2,34}$=6.052, $p<0.05$). Indeed, we found a significant decrease after the exposure to the test in both groups (no stress: -45% $p<0.05$ vs no stress/Naïve; stress reactive: -34%, $p<0.05$ vs stress reactive/Naive) as confirmed by the lack of a significant stress X test interaction ($F_{2,34}$=3.102, $p>0.05$).

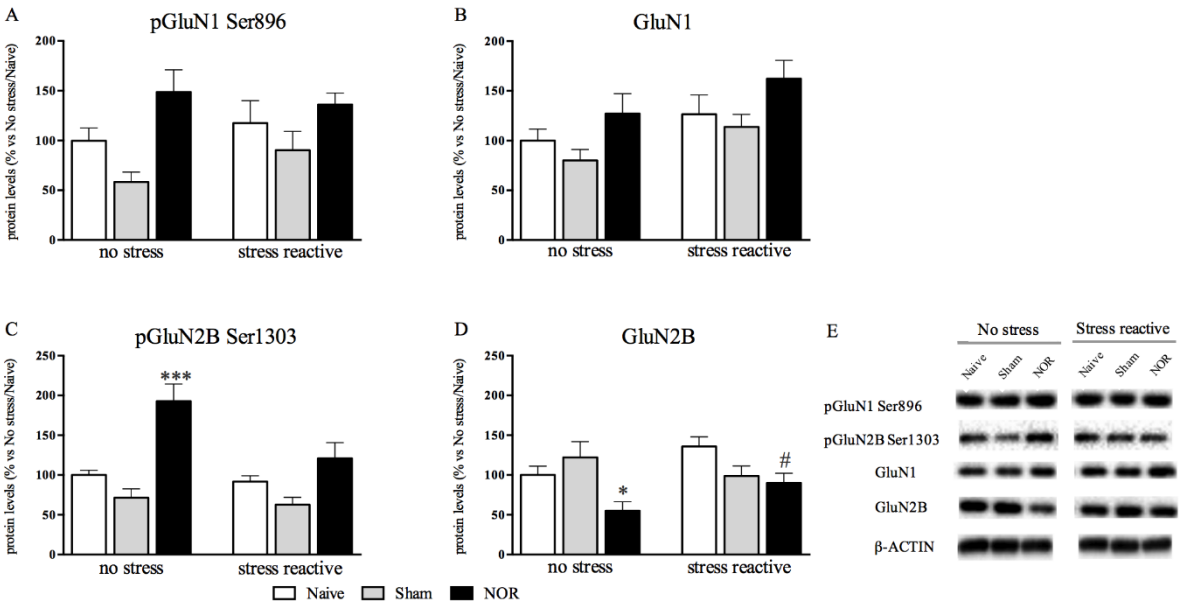

Fig. 14: Analysis of GluN1 and GluN2B, in the crude synaptosomal fraction of the dorsal hippocampus of chronically stressed rats exposed to the novel object recognition test (NOR). Phosphorylated and total levels of GluN1 and GluN2B were measured in non-stressed and chronically stressed rats exposed to the empty arena (Sham) or to the test (NOR). Panel E: Representative Western blot analyses of GluN1 (pSer896, tot), GluN2B (pSer1303, tot). β-ACTIN was used as internal standard. The data are expressed as a percentage of no stress/Naive (set at 100%). *p<0.05, ***p<0.001 vs no stress/Naive. # p<0.05 vs stress reactive /Naive (two-way ANOVA with PLSD).

1.14.1.4 Modulation of mTOR after the Novel object recognition test in the crude synaptosomal fraction of the dorsal hippocampus of control and stressed rats

One of the downstream systems activated by the NMDA receptors is mTOR. Interestingly, phospho-mTOR Ser2448 was significantly increased after the NOR test specifically in no stress animals (+44% $p<0.05$ vs no stress/Naive) (fig. 15A), but not in stress reactive rats (+0% $p<0.05$ vs stress reactive/Naive), whereas its total levels were not affected in any of the experimental groups (fig 15B). The exposure to the empty arena did not exert any significant effect (no stress: -5% $p>0.05$ vs no stress/Naïve; stress reactive: +9%, $p>0.05$ vs stress reactive /Naive), suggesting that the increased phosphorylation of mTOR on Ser2448, as well as the activation of GluN2B, may indeed be related to the cognitive performance.

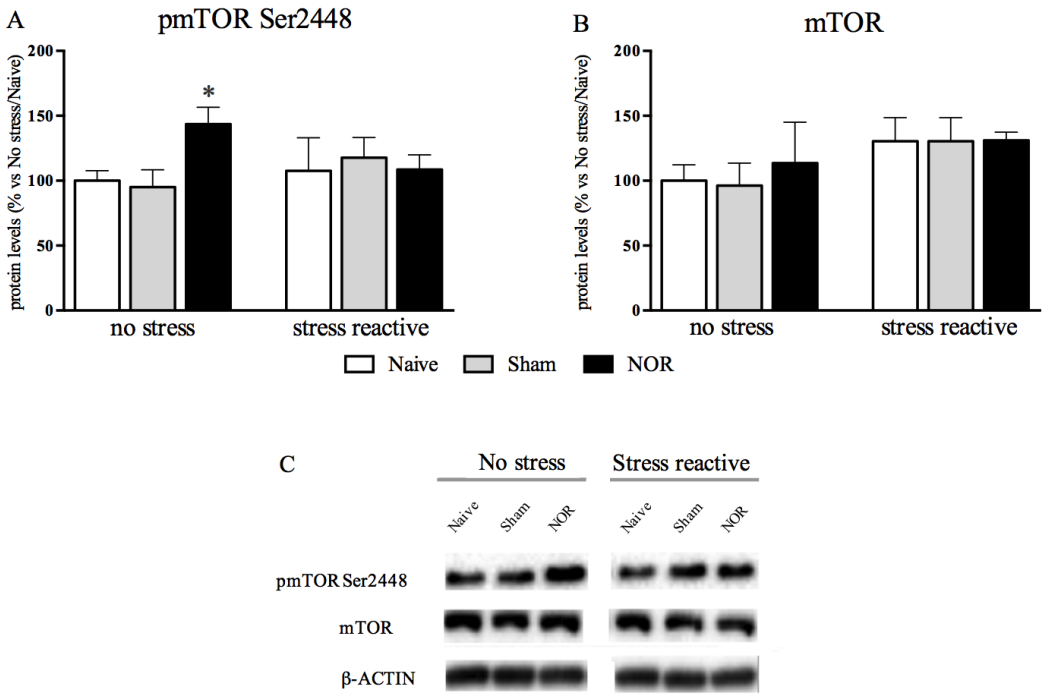

Fig. 15: Analysis of mTOR protein levels in the crude synaptosomal fraction of the dorsal hippocampus of chronically stressed rats exposed to the novel object recognition test (NOR). Phosphorylated and total levels of mTOR were measured in non-stressed and chronically stressed rats exposed to the empty arena (Sham) or to the test (NOR). The data are expressed as a percentage of no stress/Naive (set at 100%). Panel C: Representative Western blot analyses of mTOR (pSer2448, tot). β-ACTIN was used as internal standard. *$p<0.05$ vs no stress/Naive (two-way ANOVA with PLSD).

1.14.1.5 Analysis of eEF2 mediated translation in the crude synaptosomal fraction of the dorsal hippocampus of control and stressed rats

NMDAR and mTOR activation not only controls translational initiation but is also involved in peptide elongation. On these bases, we decided to analyze the phosphorylated as well as the total form of eEF2 in our experimental paradigm.

Interestingly, exposure to the cognitive test significantly increased peEF2/eEF2 (fig. 16A) in no stress (+108% $p<0.05$ vs no stress/Naive), but not in stress reactive rats (-25% $p<0.05$ vs no stress/Naive).

Since it has been previously demonstrated that the presence of uORF in the gene sequence is fundamental for the translation of the corresponding mRNAs driven by an increase of peIF2α, we explored if a similar mechanism may be associated with the phosphorylation of eEF2 (fig. 16B).

Hence, we investigated the protein levels of genes characterized by the presence of a different number of uORF, namely oligophrenin-1 (OPHN-1) (2 uORFs), BMAL-1 (7 uORFs) and ARC (0 uORFs) (fig. 16C). We found a significant increase in the OPHN1 protein levels after the NOR test, an effect that was larger in no stress (+404 $p=0.001$ vs no stress/Naïve) as compared to CMS animals (+123 $p<0.05$ vs stress reactive/Naïve) Furthermore, the protein levels of BMAL-1 were significantly up-regulated following the behavioral test in no stress rats (+68% $p<0.05$ vs no stress/Naive,) but not in CMS rats (-25% $p<0.05$ vs no stress/Naive), as suggested by the significant stressXtest interaction ($F_{1,21}=8.332$, $p<0.05$). Conversely, ARC protein levels were not affected in our experimental conditions suggesting that, similarly to what found for eIF2α, this gene-specific translational control depends on the presence of uORFs.

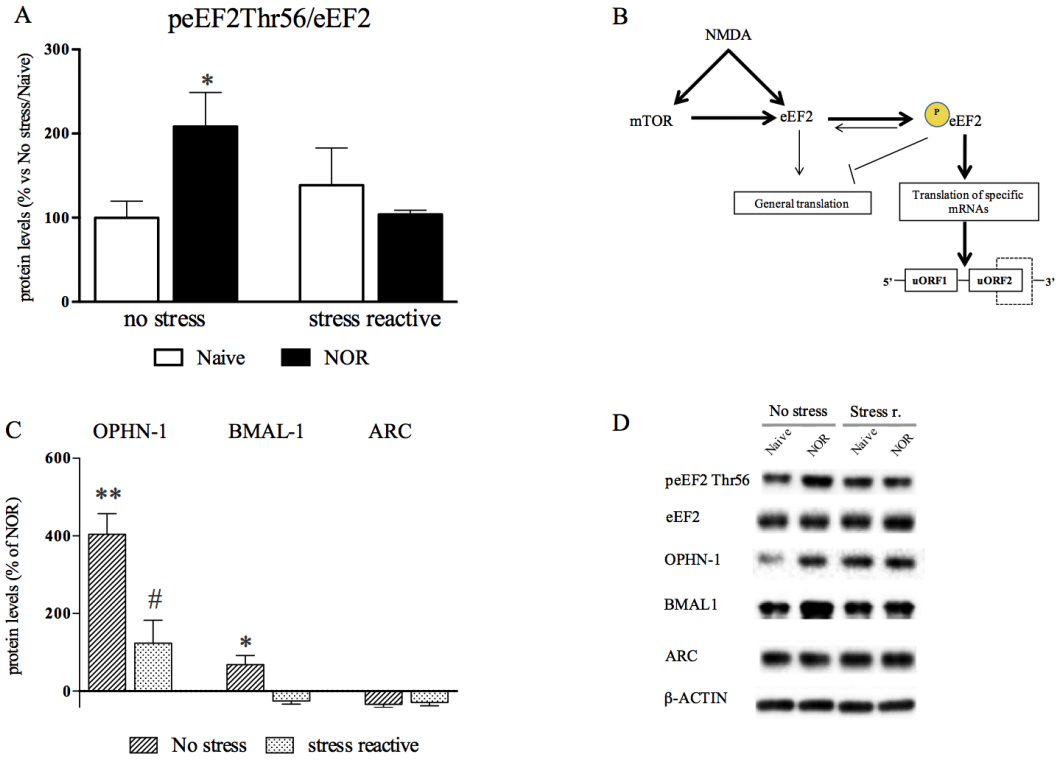

Fig. 16: Analysis of peEF2/eEF2, OPHN-1, BMAL-1 and ARC protein levels in the crude synaptosomal fraction of the dorsal hippocampus of chronically stressed rats exposed to the novel object recognition test (NOR). The data are expressed as a percentage of no stress/Naive (set at 100%). Panel B shows a schematic representation of the mechanism investigated. Briefly, we aim to investigate if the gene specific translational control by peEF2 depends on the presence of uORFs in the mRNAs sequence. Panel D: Representative Western blot analyses of peEF2 Thr56, eEF2, OPHN-1, BMAL1 and ARC. β-actin was used as internal standard. * $p<0.05$, **$p<0.01$ vs no stress/Naïve; # $p<0.05$ vs stress reactive/Naive (two-way ANOVA with PLSD).

1.14.1.6 NOR index correlates with the protein levels of pGluN2B Ser1303, pmTOR Ser2448 and peEF2/eEF2

NOR index was examined for possible covariation within the protein levels of pGluN2B, pmTOR and peEF2/eEF2. The analyses revealed that NOR index positively correlates with the expression of pGluN2B ($r = 0.7195$, $n=10$, $p<0.05$) (fig. 17A), pmTOR ($r = 0.6803$, $n=11$, $p<0.05$) (fig. 17B) and peEF2/eEF2 ($r = 0.7667$, $n=10$, $p<0.01$) (fig. 17C).

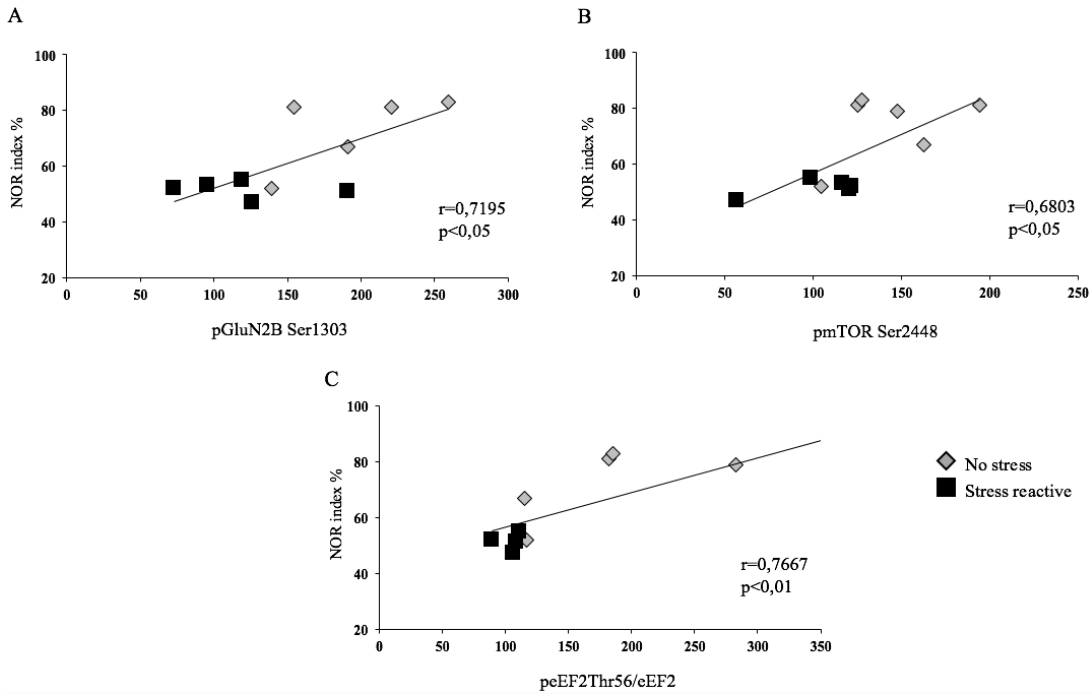

Fig. 17: *Correlation analyses between pGluN2B Ser1303 (A), pmTOR Ser2448 (B), peEF2Thr56/eEF2 (C) and NOR index in the dorsal hippocampus of non-stressed and chronically stress-reactive rats, exposed to NOR.* Analyses by Pearson's product-moment correlation (r).

1.14.2 Discussion

In this study, we showed that exposure to chronic mild stress produces cognitive deficits that are not related to the development of the anhedonic phenotype. Indeed, after 7 weeks of stress, independently from the hedonic phenotype, all the animals show cognitive deficits, as demonstrated by the reduced performance in the NOR test observed in stress reactive as well as in stress non-reactive rats (resilient to anhedonia). Moreover, our data suggest that the cognitive deficits developed following CMS are associated with an inability to activate the synthesis of new proteins specifically at the synaptic level.

Despite the fact that cognitive impairment has a disabling effect on patients and represents a major problem for the management of depressed patients, most of the studies have used stress-based models to investigate the mechanisms that contribute to anhedonia, despair, anxiety and fear, rather than focusing on cognitive deficits. Moreover, while currently available drugs may improve these symptoms of depression, cognitive deficits are not improved and may even be worsened (Millan et al., 2012).

Interestingly, at molecular levels, we found that the exposure to the NOR test, but not the novelty, induced the activation of the NMDAR in the crude synaptosomal fraction of the control rats. Conversely, but similarly to what found at the behavioral level, this effect did not occur in the stressed animals, suggesting that the cognitive deficits observed may be due to the lack of glutamate receptor activation. Accordingly, mTOR phosphorylation was specifically increased in normal animals, but not in those exposed to the chronic stress. Actually, these changes appear to be due to the cognitive test and not to a non-specific neuronal activation, since we found that the gene expression of the two activity-regulated immediate early genes, Arc and Npas4, was significantly increased after the NOR test independently from the CMS exposure and also in rats that were exposed for the same time to the empty arena (ie. Sham group).

We, thus, focused on newly synthesized proteins at synaptic levels because is controlled by NMDA and mTOR activation (Marin et al., 1997;Scheetz et al., 2000;Chotiner et al., 2003;Sutton et al., 2007;Park et al., 2008;Buffington et al., 2014) but also seen the role of this mechanism in the memory processes. Indeed, up to hundreds of mRNAs are enriched, in the dendrites of differentiated neurons, suggesting that mRNA localization is an important mechanism used to differentiate the subcellular compartments also at functional levels.

Moreover, local and specific translation of a subset of these mRNAs can allow rapid and synapse-restricted response to neuronal stimulation. Indeed, although translation occurs predominantly in the soma of the cell, the components of the translation apparatus are also found in more distal compartments of neurons, such as axons and dendrites (Steward and Schuman, 2001).

Local protein synthesis is a very complex and fine regulated mechanism (Besse and Ephrussi, 2008), associated with synaptic plasticity and memory (Kang and Schuman, 1996;Klann and Dever, 2004;Sutton et al., 2007;Gal-Ben-Ari et al., 2012;Taha et al., 2013;Buffington et al., 2014).

It is worth to mention that both NMDAR and mTOR activation not only controls translational initiation but is also involved in peptide elongation (Gauchy et al., 2002;Browne and Proud, 2004). Indeed, exposure of cortical neurons to NMDA in the absence of extracellular calcium results in increased eEF2 but not eIF2α phosphorylation (Gauchy et al., 2002). Since an increase in peEF2 has been exclusively associated with the inhibition of protein synthesis, the concomitant role of NMDARs activation in promoting translation initiation and repressing elongation may represent a paradoxical scenario. Now, we know that increased activation of peEF2 is associated with a decrease in overall protein synthesis but a concomitant increase in the protein levels of specific targets (Chotiner et al., 2003;Park et al., 2008;Buffington et al., 2014). In general, as observed for eIF2α, also the activation of eEF2 decreases general protein translation but increases the translation of specific mRNAs (Klann and Dever, 2004). Moreover, it has been (Marin et al., 1997;Scheetz et al., 2000;Sutton et al., 2007) demonstrated that NMDA-mediated elongation might be considered a more finely regulated mechanism that contributes to the proper timing and spatial localization of neuronal translational events (Marin et al., 1997;Scheetz et al., 2000;Sutton et al., 2007).

Moreover, in neurons, eEF2 is regulated in a compartment-specific fashion. Indeed, Sutton and colleagues found that NDMAR-dependent miniature synaptic events in hippocampal neurons increase dendritic peEF2 (Sutton et al., 2007). Accordingly, *in vivo*, there are higher levels of peEF2 in synaptoneurosomal fraction than in total cell homogenate (Belelovsky et al., 2005) and in dissociated hippocampal neurons, long-term treatment with tetrodotoxin or bicuculline has opposing effects on peEF2 in dendrites compare to the soma (Verpelli et al., 2010).

In line with the activation of the NMDA/mTOR pathways following the behavioral test in control rats, we found that the peEF2/eEF2 ratio was significantly increased in no stress rats, suggesting possible changes in the translation of specific mRNAs. Accordingly, the involvement of eEF2 in memory and learning was already reported in different taste-learning paradigms in physiological conditions (Gal-Ben-Ari et al., 2012;Taha et al., 2013). Moreover, chronic administration of the selective serotonin reuptake inhibitor, fluoxetine, induced eEF2 phosphorylation in the prefrontal cortex, hippocampus and dentate gyrus (Dagestad et al., 2006).

Interestingly, after the NOR test, we found an increase of OPHN-1 and BMAL1 in no stress rats, but not in CMS rats, while ARC protein levels were unchanged, suggesting that the gene-

specific translation control by peEF2, as indicated for peIF2α, depends on the regulatory elements in the mRNA namely open reading frames (uORFs).

Supporting the role of Ophn-1 in cognitive processes, it has been recently demonstrated that its silencing in the hippocampus leads to impairment in the novel object recognition test (Di Prisco et al., 2014). Moreover, it has been reported that, at synaptic levels, oligophrenin-1 interacts with Rev-erbα (Valnegri et al., 2011). Since both Bmal1, as Rev-erbα, belongs to the circadian clock and are involved in the controls of circadian rhythmicity (Lowrey and Takahashi, 2011), our results confirm the hypothesis of Valnegri et al. on the influence of circadian rhythms on cognitive ability.

Interestingly, we found a positive and significant correlation between the protein levels of pGluN2B, pmTOR and peEF2/eEF2 and the NOR index suggesting that deficits in these 'synaptic' mechanisms may indeed contribute to the cognitive impairment observed in CMS rats.

In summary, our results suggest that the correct performance in a cognitive test is associated with the translation of specific mRNAs at synaptic levels and that the cognitive deficits due to chronic stress exposure are due to alterations of this mechanism. Moreover, we highlighted a fundamental role of the elongation step in the correct cognitive performance. It may be inferred that pharmacological intervention able to normalize these alterations might improve cognitive function in patients with major depression and stress-related disorders.

1.15 Effect of prolonged lurasidone treatment on chronic mild stress-induced alterations: a role for glucocorticoid receptor

Manuscript in preparation

It is well known that psychiatric diseases are characterized by an altered function of the hypothalamic-pituitary-adrenal axis (Holsboer, 2000;de Kloet et al., 2005).
In particular, the HPA axis is controlled by a feedback mechanism that serves as negative regulator of the axis' activity. This system is deregulated in stress-related disorders, including major depression, with a disruption of the feedback that lead to high levels of circulating cortisol and to an excessive activation of the HPA axis.
Moreover, since a correct hormonal response is essential for learning and memory processes (Sandi et al., 1997) the alterations of this system may contribute to the development of cognitive deficits that represents one of the most debilitating symptoms of major depression. Indeed, cognitive impairment may persist even when patients are successfully treated with antidepressants and remission is achieved, thus representing a residual symptom that reduces the everyday performance and causes considerable distress.
Glucocorticoids hormones act via genomic mechanisms, involving nuclear receptors, as well as via non-genomic pathways that require membrane-associated receptors.
In particular, the genomic action of GRs regulates the transcription of target genes that contain in the promoter the glucocorticoid responsive element, including genes playing a key role in synaptic plasticity and memory (Datson et al., 2001;Morsink et al., 2006;Datson et al., 2008). Furthermore, in the non-genomic pathways, GCs can directly stimulate the release of excitatory amino acids, via the synaptic membrane associated receptors, and can regulate mitochondrial oxidation and free radical formation through the binding with GRs on the mitochondrial membranes (McEwen et al., 2015).
In the present study, we investigated the changes occurring in the HPA axis following chronic stress and their potential involvement in the anhedonic phenotype as well as in the cognitive impairment that develop in animals vulnerable to stress exposure (Calabrese et al., 2017). Moreover, we evaluated the ability of chronic lurasidone treatment in counteracting the behavioral impairment observed in CMS rats and the possible role of the GRs in its effect. The molecular analyses were conducted in the dorsal hippocampus, a brain structure highly sensitive to stress, which plays an important role in memory formation (Kim and Diamond, 2002).

1.15.1 Results

1.15.1.1 Behavioral characterization of chronically stressed rats treated with lurasidone

As previously demonstrated (Calabrese et al., 2017), already after one week of chronic stress exposure, rats developed an anhedonic phenotype, as indicated by the reduced consumption of 1% sucrose solution (-7gr, $p<0.001$ vs no stress/VEH), an effect that persisted for the subsequent 6 weeks of CMS (week2: -7.6gr, $p<0.001$ vs no stress/VEH; week3: -6 gr, $p<0.001$ vs no stress/VEH; week4: -7.9, $p<0.001$ vs no stress/VEH; week5: -7.5, $p<0.001$ vs no stress/VEH; week6: -7.4gr, $p<0.001$ vs no stress/VEH; week7: -6.8gr, $p<0.001$ vs no stress/VEH).

After the initial two weeks of CMS protocol, animals that had developed anhedonia were randomized in two groups to receive vehicle or lurasidone for five weeks, while continuing stress exposure.

Chronic treatment with lurasidone, in comparison to vehicle administration, did not affect sucrose intake in control animals, while starting from the second week of treatment (fifth week of stress protocol), the drug significantly increased the sucrose intake in stressed animals (week5: +3.2 gr, $p<0.05$ vs CMS/VEH; week6: +4gr, $p<0.05$ vs CMS/VEH; week7: +5gr, $p<0.05$ vs CMS/VEH) (fig. 18A). As clearly shown in figure 18B, by quantifying the overall result after 7th weeks of CMS, we found a significant effect of stress ($F_{1\text{-}80}=19.256$, $p<0.001$), of the treatment ($F_{1\text{-}80}=4.322$, $p<0.05$) and a stressXtreatment interaction ($F_{1\text{-}80}=16.724$, $p<0.001$). Indeed, stress exposure significantly decreased sucrose consumption (-6.8gr, $p<0.001$ vs no stress/VEH), effect that was normalized by the lurasidone administration (+5gr, $p<0.001$ vs CMS/VEH).

Interestingly, in line with previous findings (Calabrese et al., 2017), 7 weeks of CMS led to the development of cognitive deficits, as indicated by the significant reduction of the NOR index (-30%, $p<0.001$ vs no stress/VEH). When examining the effect of lurasidone on stress-induced deficits in cognition, two-way ANOVA analysis revealed a significant effect of CMS ($F_{1\text{-}40}=9.869$, $p<0.01$), of treatment ($F_{1\text{-}40}=9.688$, $p<0.01$) as well as a stressXtreatment interaction ($F_{1\text{-}40}=6.866$, $p<0.05$). Indeed, the pharmacological treatment completely restored the stress mediated-alterations (+42%, $p<0.001$vs no CMS/VEH) (fig. 18C).

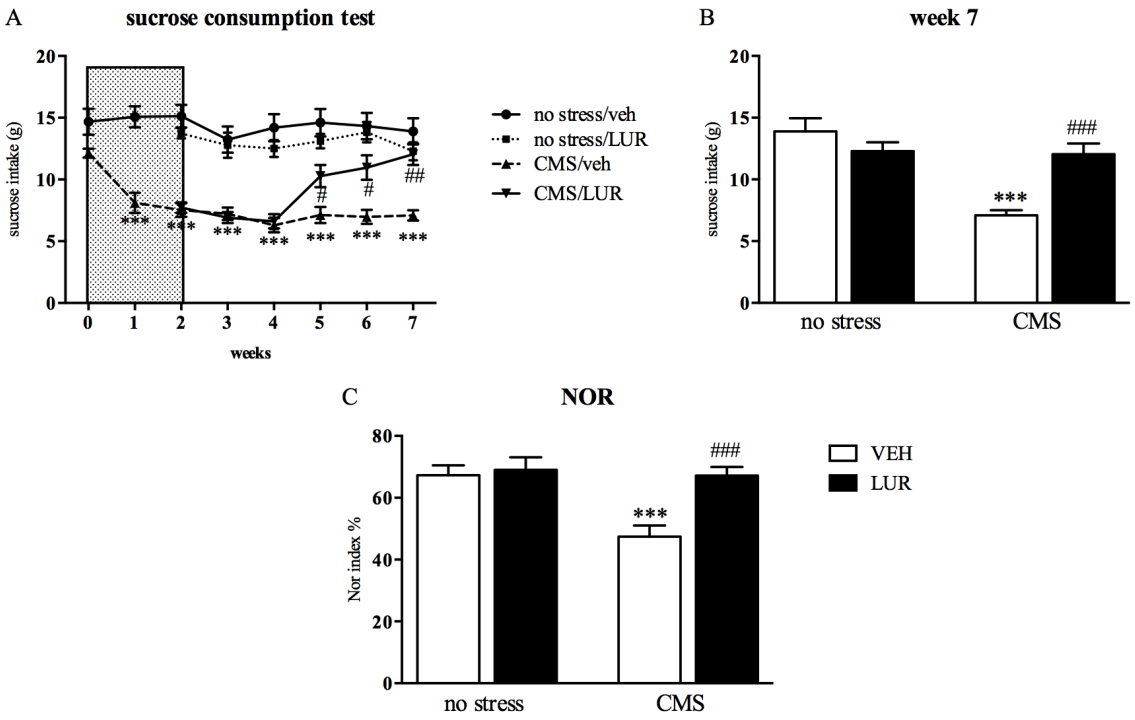

Fig. 18: Behavioural characterization of animals exposed to chronic mild stress (CMS) and treated with lurasidone (LUR). The data are the mean ± SEM of at least 10 animals. Panel A shows the sucrose intake performed every week, while panel B reports the results of the test after 7 weeks of CMS, expressed as grams of consumed sucrose. The result of the novel object recognition test is summarized in the panel C. ***p<0.001 vs no stress/VEH; #p<0.05, ##p<0.01; ### p<0.001 vs CMS/VEH) (two-way ANOVA with repeated measures panel A; two-way ANOVA with PLSD panel B-C).

1.15.1.2 Modulation of nuclear GR protein levels and of GR responsive genes by chronic stress exposure

In order to establish whether GR genomic effect contribute to the cognitive performance, we evaluated nuclear GR signaling both under resting condition and following the exposure to the novel object recognition test in animals expose or not to the CMS paradigm.

GR receptor in the nuclear compartment was significantly modulated by the stress ($F_{1,41}$= 4.342, $p<0.05$), by the test ($F_{1,41}$= 10.624, $p<0.01$) and by the stressXtest interaction ($F_{1,64}$= 7.106, $p<0.05$) as revealed by the two-way ANOVA. Indeed, we found that exposure to NOR test significantly increased nuclear GR protein levels in non-stressed animals (+96%, $p<0.001$ vs no stress/Naïve), an effect that that was not observed after the task in CMS rats (+9%, $p>0.05$ vs no stress/Naïve) (fig. 19A). These results suggest that the cognitive test requires a significant translocation of GR into the nucleus, a mechanism that is impaired in CMS rats. Hence, in order to establish if the translocation of GRs to the nuclear compartment is associated with the transcription of genes containing the GRE in their sequences, we measured the mRNA levels of some GR responsive genes, which are known to play a role in stress response and may be dysregulated in psychiatric disorders. Interestingly, the modulation of nuclear GR levels was paralleled by an upregulation of different GR responsive genes, such as *Gadd45β*, *Sgk-1* and *Nr4a1*, involved in learning and memory. For *Gadd45β* we found a significant effect of CMS ($F_{1,64}$= 6.926, $p<0.05$), of the cognitive test ($F_{1,64}$= 20.461, $p<0.001$) as well as a stressXtest interaction ($F_{1,64}$= 7.333, $p<0.01$). Indeed, we found that non-stressed rats exposed to the NOR test showed a significant increase of *Gadd45β* mRNA levels (+96%, $p<0.001$ vs no stress), as compared to naïve rats, an effect that that was not observed in CMS rats exposed to the behavioral test (fig. 19B). As shown in figure 19C, two-way ANOVA analysis revealed a significant stress ($F_{1,62}$= 8.141, $p<0.01$) and test ($F_{1,62}$= 14.625, $p<0.001$) effect on *Sgk1*, with its expression being up-regulated by the test only in non-stressed rats (31%, $p<0.001$ vs no stress/Naïve). Similarly, *Nr4a1* was significantly affected by the test ($F_{1,38}$= 5.605, $p<0.05$) with an increase due to the behavioral task (+26%, $p<0.05$ vs no stress/Naïve) in control animals (fig. 19D).

As shown in figure 19E, we observed a significant effect of the behavioral test ($F_{1,39}$= 28.408, $p<0.001$) on *Dusp1* expression. Indeed, independently by stress exposure, the gene was up-regulated by the NOR both in non-stressed (+38%, $p<0.05$ vs no stress/Naïve) and in stressed rats (+29%, $p<0.05$ vs CMS/Naïve).

Of note, other GR-related genes analyzed, such as *Fkbp5* and *P11* were not altered by CMS or NOR (fig. 19F-G).

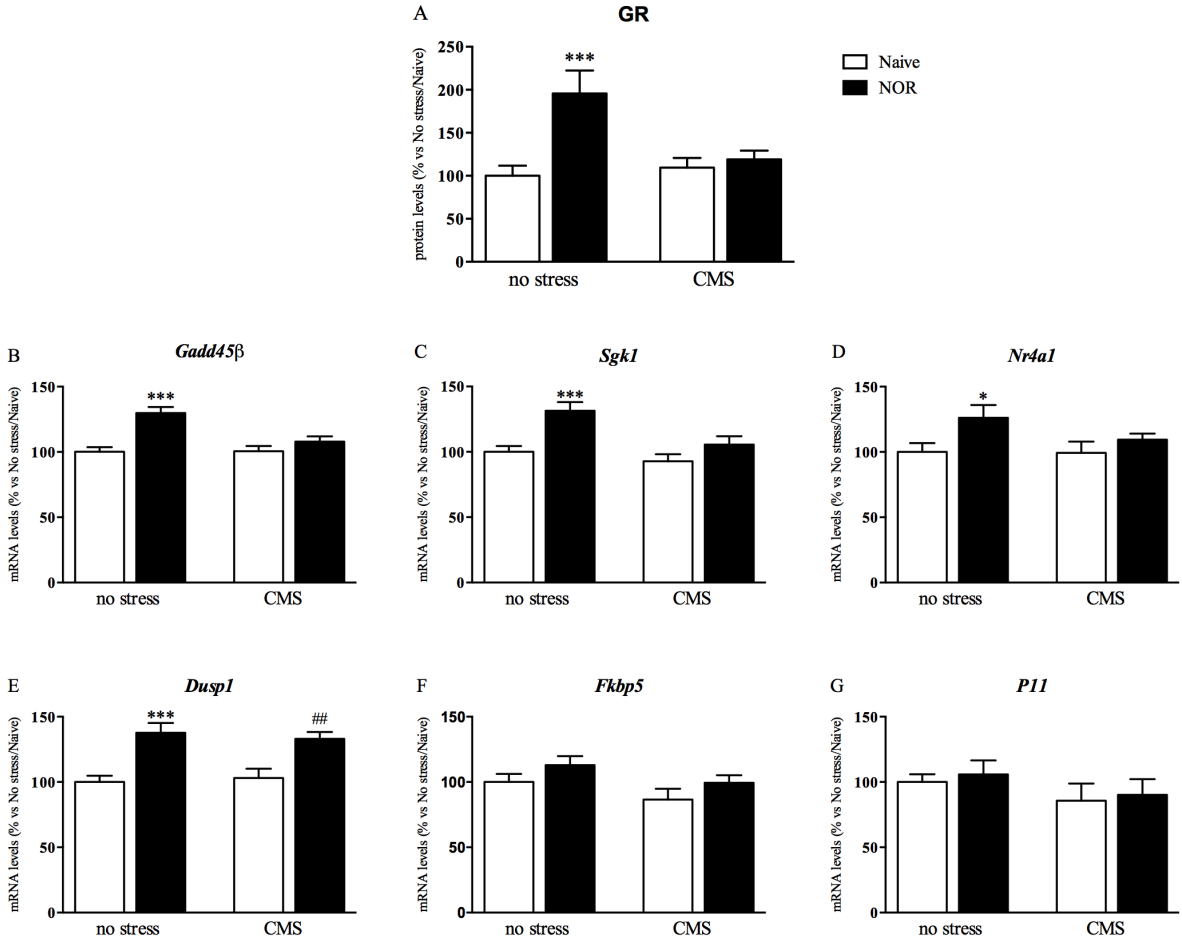

Fig. 19: Analysis of nuclear GR protein levels and of GR-responsive gene expression in the dorsal hippocampus of chronically stressed rats, under resting conditions or after exposure to the novel object recognition test (NOR). The data, expressed as the percentage of no stress/Naive (set at 100%), are the mean ± SEM of at least 6 animals. * $p<0.05$, ***$p<0.001$ vs no stress/Naive, ## $p<0.01$ CMS/Naive (two-way ANOVA with PLSD).

1.15.1.3 Modulation of nuclear GR protein levels and of GR-responsive genes by lurasidone treatment in chronically stressed rats

We next examined the modulation of GR levels in the nuclear compartment in CMS rats treated with lurasidone and exposed to the NOR test, as compared to CMS rats treated with vehicle, in order to assess if the pharmacological treatment was able to exert its beneficial activity at behavioral level by restoring the GR genomic pathways, altered by chronic stress.

As shown before (fig. 19A), exposure to CMS prevents the translocation of GR in the nucleus following the cognitive test. Interestingly CMS rats treated with lurasidone showed a significant increase of nuclear GR levels upon exposure to the NOR (+36%, $p<0.05$ vs CMS/VEH), as indicated by the two-way ANOVA results ($F_{1,30}= 5.060$, $p<0.05$), an effect that resembles what we have observed in non-stressed rats during the cognitive performance (see above, fig. 19A).

Moreover, lurasidone treatment in CMS rats was able to facilitate the transcription of the GR responsive genes *Gadd45β*, *Sgk1, Nr4a1* and *Dusp1* following the NOR test. Indeed, we found a significant effect of the treatment (*Gadd45β:* $F_{1,52}= 7.200$, $p<0.05$; *Sgk1:* $F_{1,50}= 6.672$, $p<0.05$), of the test (*Gadd45β:* $F_{1,52}= 16.252$, $p<0.001$ *Sgk1:* $F_{1,50}= 21.344$, $p<0.001$) and a significant treatmentXstress interaction (*Gadd45β:* $F_{1,52}= 7.200$, $p<0.05$; *Sgk1:* $F_{1,50}= 6.571$, $p<0.05$), with an up-regulation of *Gadd45β* (fig. 20B) and *Sgk1* (fig. 20C) upon exposure to the NOR test only in CMS animals that were chronically treated with lurasidone (+39%, $p<0.001$ vs CMS/LUR/Naïve; +49%, $p<0.001$ vs CMS/LUR/naïve respectively).

With respect to *Nr4a1,* two-way ANOVA revealed a significant effect of the treatment ($F_{1,39}= 5.741$, $p<0.05$) and of the NOR test ($F_{1,39}= 5.412$, $p<0.05$). Indeed, *Nr4a1* was upregulated (+46%, $p<0.05$ vs CMS/LUR/Naïve) only in CMS/LUR/NOR group (fig. 20D).

Moreover, as shown in figure 20E, *Dusp1* was increased by the NOR independently from the pretreatment both in vehicle (+29%, $p<0.01$ vs CMS/VEH/Naïve) and in lurasidone (+22%, $p<0.01$ vs CMS/LUR/Naïve) treated rats, as confirmed by the results of the two-way ANOVA (treatment: $F_{1,39}= 6.685$, $p<0.05$; NOR test: $F_{1,39}= 21.169$, $p<0.001$, treatmentXtest interaction: $F_{1,39}= 0.107$, $p>0.05$).

On the contrary, Fkbp5 and P11 expression was not modulated in all the experimental groups nor by the treatment neither by the test (fig 20F-G).

These results suggest that the correct cognitive performance requires the activation of the transcription of genes controlled by the GR genomic pathway. Accordingly, the deficit in the cognitive task caused by chronic stress is associated with an alteration of these mechanisms. Interestingly, lurasidone normalized the cognitive deficits due to CMS exposure (fig. 18C) by restoring the ability of GR to translocate in the nucleus and regulate the expression of key genes.

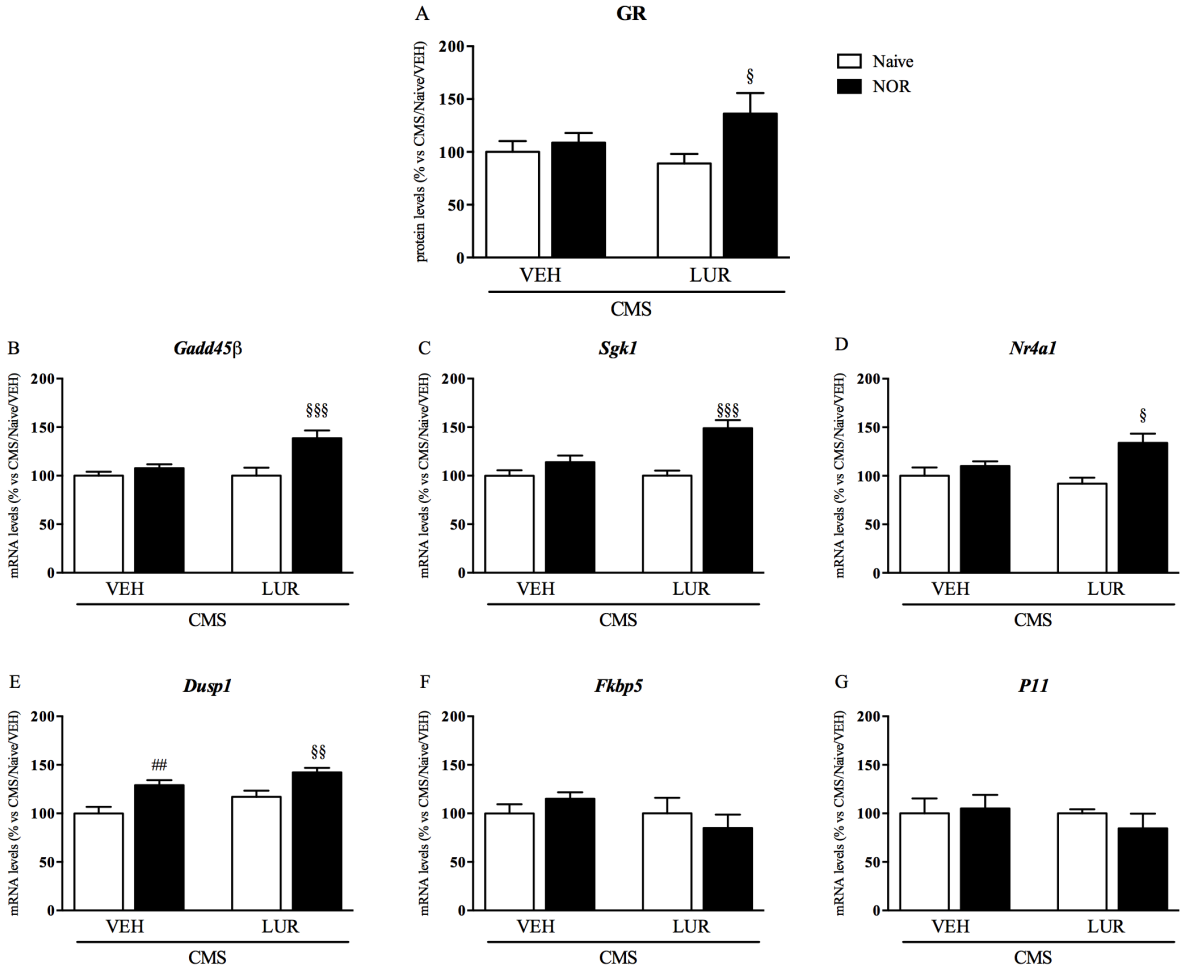

Fig. 20: Analysis of nuclear GR protein levels and of GR-responsive gene expression in the dorsal hippocampus of chronically stressed rats treated with lurasidone, under resting conditions or after exposure to the novel object recognition test (NOR). The data, expressed as the percentage of no stress/Naive (set at 100%), are the mean ± SEM of at least 6 animals. ## p<0.01 CMS/Naïve/VEH; §p<0.05, §§p<0.01, §§§ p<0.001 vs CMS/Naïve/LUR (two-way ANOVA with PLSD).

1.15.1.4 Modulation of GR non genomic pathway by chronic stress exposure

Next, we investigated the impact of the chronic stress exposure on the GR non-genomic mechanisms in naïve animals as well as in rats tested to the NOR.

As shown in figure 21A, exposure to CMS produced a significant increase of GR protein levels in the membrane fraction (+60% $p<0.05$ vs no stress/Naïve), as revealed by the two-way ANOVA results on stress effect ($F_{1\text{-}27}=7.440$, $p<0.05$). Moreover, as confirmed by the non-significant effect of the cognitive test in the two-way ANOVA analysis ($p>0.05$), the exposure to the NOR did not alter GR expression at membrane level, nor in control neither in stressed groups.

The activity of membrane-bound GRs, as described above, involves both synaptic as well as mitochondrial mechanism.

In order to investigate the potential functional activity of GRs in the synaptosomal membranes we measured SYNAPSIN-I protein levels, whereas for the mitochondrial counterpart we assessed the expression of the subunits 1 (cytochrome oxidase 1, Cox1) and 3 (cytochrome oxidase 3, Cox3) of the cytochrome c oxidase, a key enzyme in the mitochondrial respiratory chain.

In line with the increase 'membrane-bound' GRs observed in stressed rats, as shown in figure 21B, we found a significant effect of CMS exposure on phospho Ser603 SYNAPSIN-1 ($F_{1\text{-}30}=11.357$, $p<0.01$). Indeed, its levels were up-regulated (+88%, $p<0.05$ vs no stress/Naive) in stressed rats compared to naïve counterpart. These changes were not associated with significant alterations of total SYNAPSIN-1 levels (fig. 21C).

Regarding mitochondrial mechanisms, as suggested by the significant effect in the two-way ANOVA ($F_{1\text{-}39}=6.406$, $p<0.05$), chronic stress exposure produced an increase of *Cox3* expression (+21%, $p<0.05$ vs no stress/Naive) (fig 21E), as compared to non-stressed animals, whereas we did not observe any statistically significant change for *Cox1* mRNA levels (fig. 21D).

Furthermore, exposure to the NOR test did not induce any significant modification nor of the membrane-bound GR protein levels, neither of the synaptic and mitochondrial downstream effectors (fig. 21, panel A,B,C,D,E), indicating that all the modulations observed in the non-genomic pathway were mainly driven by the chronic stress, independently from the cognitive task exposure.

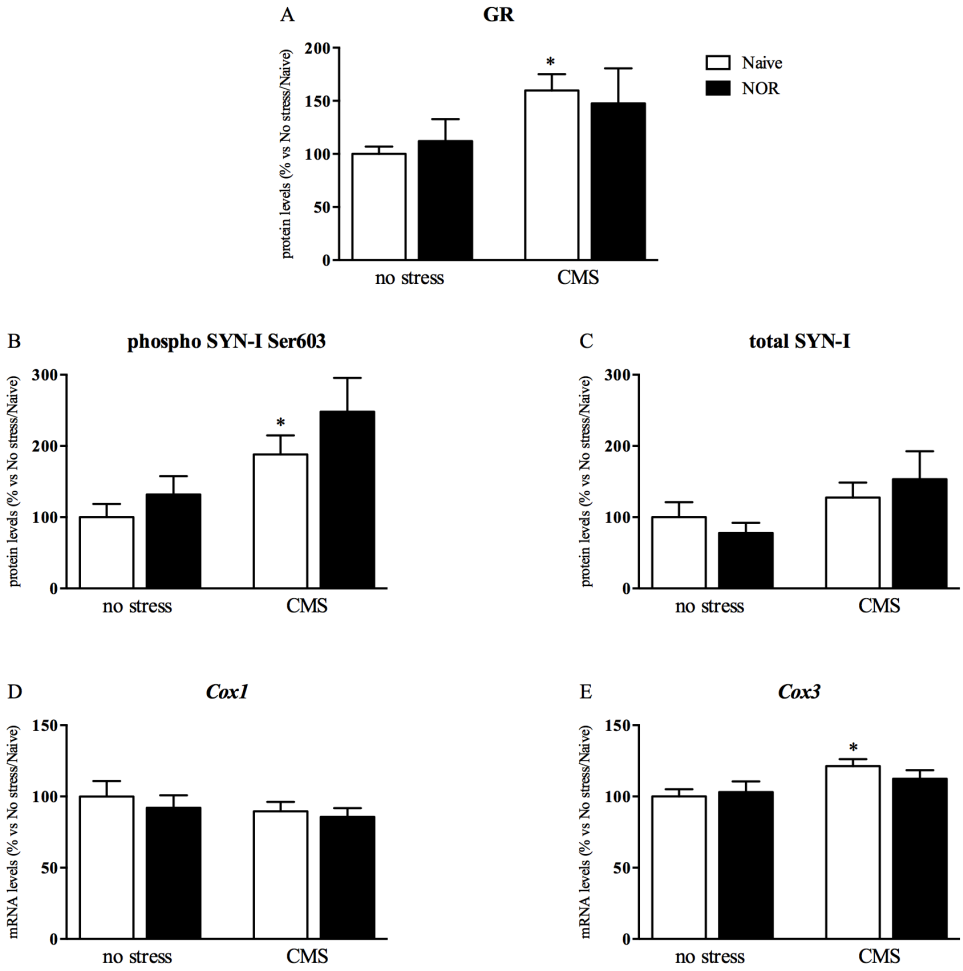

Fig. 21: Analysis of membrane-bound GR, phospho SYN-I Ser603 protein levels and Cox1 and Cox3 mRNA levels in the dorsal hippocampus of chronically stressed (CMS) rats exposed to the novel objet recognition test (NOR). The data, expressed as the percentage of no stress/Naive (set at 100%), are the mean ± SEM of at least 6 animals. * $p<0.05$ vs no stress/Naive (two-way ANOVA with PLSD).

1.15.1.5 Modulation of GR non genomic pathway by lurasidone treatment in chronically stressed rats

Last, we investigated the impact of the pharmacological intervention with lurasidone on membrane-bound GR mechanisms. As shown in figure 22A, we observed a significant effect of stress (F_{1-25}=5.880, p<0.05), of treatment (F_{1-25}=4.879, p<0.05) as well as a stressXtreatment interaction (F_{1-25}=5.880, p<0.05) for GR protein levels. Indeed, the significant increase of membrane GR levels due to CMS exposure (+60% p<0,001 vs no stress/VEH) was normalized by the chronic lurasidone treatment (-36%, p<0,01 vs. CMS/VEH).

Accordingly, the increased of phospho SYN-1 Ser603 (fig. 22B) observed after CMS (+88%, p<0.01 vs CMS/VEH) was completely reverted by the pharmacological administration (-58%, p<0,01 vs CMS/VEH), as supported by the significant stressXtreatment interaction (F_{1-29}=8.007, p<0.01) in the two-way ANOVA analysis. Conversely, the treatment did not significantly modulate the total form of SYN-I.

With respect to the mitochondrial targets, the *Cox1* expression was modulated by the treatment (F_{1-40}=5.880, p<0.05). Indeed, lurasidone downregulated the mRNA levels specifically in non-stressed animals (-21%, p<0.05 vs no stress/VEH) (fig. 22D). On the contrary, as shown in figure 21E, we found a significant effect of the stress (F_{1-39}=4.434, p<0.05) and of the treatment (F_{1-39}=4.434, p<0.05) for the *Cox3*, with an increase of its expression due to CMS exposure (+21%, p<0.05 vs no stress/VEH) being normalized by prolonged lurasidone treatment (-20%, p<0.01 vs CMS/VEH).

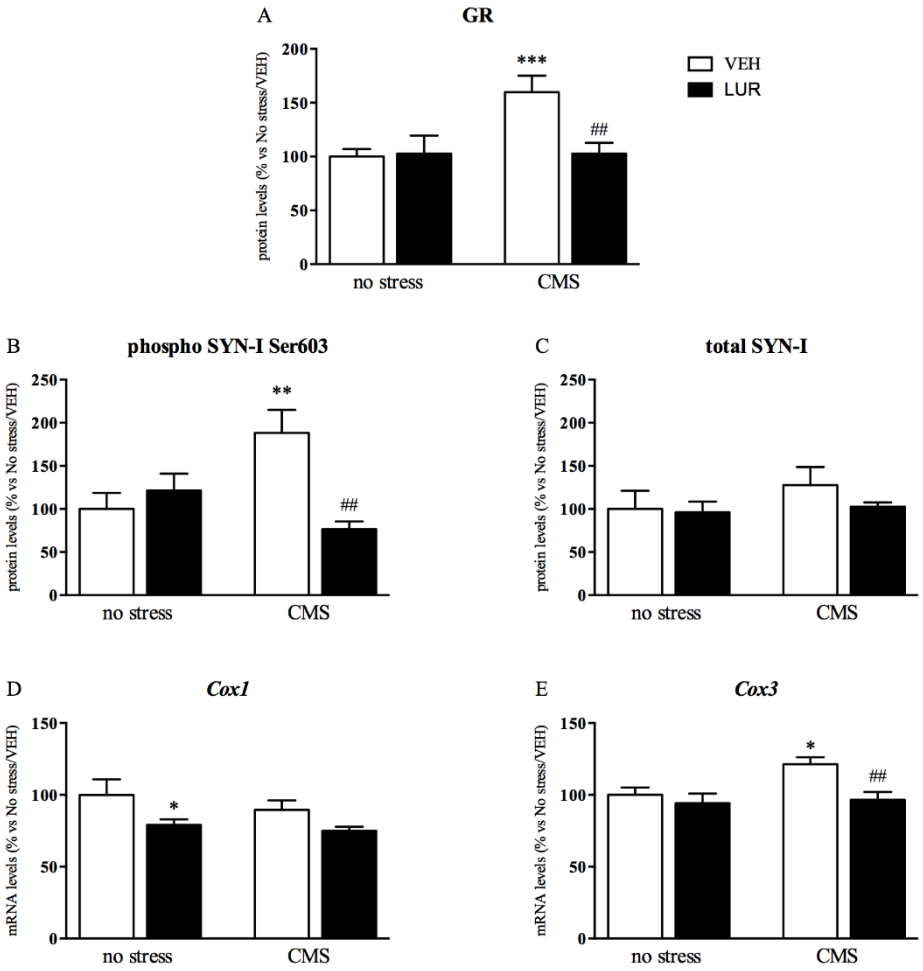

Fig. 22: Analysis of membrane GR, phospho SYN-I Ser603 protein levels and Cox1 and Cox3 mRNA levels in the dorsal hippocampus of chronically stressed (CMS) rats treated with lurasidone (LUR). The data, expressed as the percentage of no stress/Naive (set at 100%), are the mean ± SEM of at least 6 animals. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs no stress/VEH; ## $p<0.01$ vs CMS/LUR (two-way ANOVA with PLSD).

1.15.2 Discussion

In this study, we demonstrated that the cognitive deficit caused by the exposure to the CMS is associated with an altered activity of both the genomic and of the non-genomic GR pathways. Moreover, we found that the ability of prolonged lurasidone treatment to normalize chronic mild stress induced-behavioral alterations is accompanied by a normalization of all the GR-related alterations.

Interestingly, besides the well-established rescue of the anhedonic phenotype (Calabrese et al., 2016;Rossetti et al., 2016), lurasidone treatment was also able to normalize the cognitive deficits produced by chronic stress exposure.

Glucocorticoid receptor in the brain are essential players for the response to stressful situations, both in term of mood alterations as well as well as for the regulation of memory-related mechanisms (Sapolsky et al., 2000), playing a critical role, both in rodents and in human, in encoding processing and retaining information of emotional events (Lupien et al., 2007;McIntyre et al., 2012). Indeed, whereas low-moderate levels of stress stimulate cognitive performances and memory formation, severe and chronic stressful experiences can lead to cognitive impairments and to the development of psychopathologies (de Kloet et al., 2005;McEwen et al., 2015).

The stress response is finely regulated by GCs through complex mechanisms that involve different pathways. In particular, GRs are localized in different subcellular compartments thus mediating several effects by acting at genomic and non-genomic levels. Accordingly, GCs' actions are critical depending on the timing and on the level of GR expression (Joels et al., 2006).

Here, we found that a correct cognitive performance is associated with the activation of the genomic pathway, as revealed by the enhanced translocation of the receptor to the nuclear compartment and the transcription of some GR-regulated genes, including *Gadd45β*, *Sgk1*, *Nr4a1* and *Dusp1* that may facilitate the cognitive performance, since, these GR targets are known to be implicated in memory and learning processes. For example, it has been shown that Gadd45β KO mice exhibited deficits in the hippocampal long-term memory (Leach et al., 2012), whereas elevated levels of Sgk and Nr4a1, known to promote memory formation (Hawk and Abel, 2011), were found in the hippocampus of rats after learning tasks (Pena de Ortiz et al., 2000;Tsai et al., 2002;von Hertzen and Giese, 2005) and transfecting Sgk in the CA1 hippocampal subfield facilitated spatial memory performance in rats (Tsai et al., 2002). Moreover, Dusp1 plays an important role in modulating cellular response to environmental stress as an immediate early response gene (Noguchi et al., 1993;Sun et al., 1993).

The observation that the induction of nuclear mechanism of GRs in the dorsal hippocampus during the cognitive test was completely blunted in stressed rats further supports the role of genomic mechanism for the correct performance in the NOR, and it is in line with the findings that chronic stress protocols determine important deficits in hippocampal-dependent form of memory (Luine et al., 1993;Luine et al., 1994;Yuen et al., 2012).

Furthermore, we found that 7 weeks of chronic mild stress strongly increased the membrane-bound GRs that may have implications for synaptic mechanisms and mitochondrial function. In particular, the up-regulation of GR protein levels at membrane levels was paralleled by an up-regulation of the active form of SYNAPSIN 1, a marker of the functional activity of the receptor in the synaptosomal membranes. Accordingly, 21 days of CUS significantly increased the density of SYNAPSIN 1 immunoreactive synaptic buttons in the CA3 subfield of the rat hippocampus, effect that was restored by the treatment with the GR antagonist Mifepristone (Wu et al., 2007). Moreover, since rat model of depression induced by CUS are characterized by the corticosterone hypersecretion (Ayensu et al., 1995), these findings are in accordance with the report that corticosterone increases the amount of SYNAPSIN I in rat hippocampus (Nestler et al., 1981).

Furthermore, glucocorticoids may also act on GR located on mitochondria membrane and regulate the translation of genes involved in the respiratory enzyme biosynthesis (Tsiriyotis et al., 1997), such as *Cox1* and *Cox3*, the catalytic subunits of cytochrome c oxidase, the last enzyme in the respiratory electron transport chain (Demonacos et al., 1996;Liang et al., 2006). Here we found that chronic stress exposure produced an up-regulation of *Cox3* mRNA levels, but not of the *Cox1,* in the dorsal hippocampus. In line with the translocation of GR into mitochondria under conditions of stress or elevated corticosteroid levels, the expression of genes, including *Cox1* and *Cox3,* have been found to be upregulated in the rat hippocampus by injection of 300 μg/kg corticosterone (Hunter et al., 2016), whereas Adzic and colleagues found a decreased expression of *Cox1* and *Cox3* in the whole hippocampus, and an increase in the prefrontal cortex of rats exposed to 21 days of chronic restraint stress (Adzic et al., 2009).

Interestingly, lurasidone treatment exerted its beneficial activity at behavioral level, by restoring both the anhedonic phenotype and the cognitive alterations, probably acting at molecular level by facilitating the nuclear GR translocation, by regulating the concomitant transcription of its key genes during ongoing cognitive activity and by normalizing the membrane GR content, thus restoring the normal functioning of both phospho SYN-1 Ser603 and *Cox3* gene. These results indicated the useful role of lurasidone in counteracting the negative effect driven by chronic stress in the non-genomic mechanisms of GR.

In summary our findings suggest that the activation of the genomic pathway mediated by GR may contribute to the correct cognitive performance, while chronic stress exposure interferes

with this mechanism. Moreover, CMS, increasing the availability of GR at membrane levels, seems to direct preferentially the action of hormones more towards the non-genomic pathways, thus altering synaptic and mitochondrial signaling. In particular, the behavioral deficits we observed may be related with both the altered genomic and non-genomic mechanism of GR and the dysregulations of these signaling in stressed rats might be indicative of the so-called "glucocorticoid resistant" a key feature of depressed patients.

In addition, we highlight the ability of lurasidone in normalizing the behavioral outcomes, induced by CMS exposure, by restoring the modification observed in the GR mediated effects, suggesting the potential ability of the drug in modulating dysfunction related with the HPA axis. These data provide new insights on the mechanism of action of lurasidone in the context of stress-related disorders, indicating that its pharmacological profile may be responsible for peculiar adaptive mechanisms that may be critical for the ability to modulate different pathologic domains associated with psychiatric disorders.

1.16 Effects of chronic stress exposure and lurasidone treatment on HPA axis function: focus on DNA methylation

Unpublished data

DNA methylation of cytosines in cytosine-guanine (CpG) dinucleotides is one of the major form of epigenetic modifications that regulates gene expression by affecting the binding of transcription factor and regulatory elements (Razin and Riggs, 1980;Bird, 1986). In particular, DNA methylation machinery establishes specific methylation patterns during both development and adulthood in response to environmental signals and maintains these modifications during cell division and after DNA repair (McGowan and Szyf, 2010).

The epigenetic effects of the environmental stressors have been identified as risk factors for different psychiatric disorders, including MDD. Epigenetics are mechanisms that in response to social and physical environmental factors, including adverse stimuli, can result in lasting changes that affect brain functions and neurobiological processes, including the neuroendocrine system (Auger and Auger, 2013). They constitute important mechanisms by which transient stimuli can induce persistent changes in gene expression and in behavior (Szyf et al., 2008;Zovkic et al., 2013).

Moreover, many antidepressant drugs have been found to influence epigenetic processes, by acting as regulators of key mechanisms, thus exerting beneficial effects (Frieling and Tadic, 2013).

As previously mentioned, MDD is associated with functional alterations of the HPA axis and the gene encoding for the glucocorticoid receptor, Nr3c1, undergoes changes in the methylation of its promoter in the context of environmental adversities.

For example, it has been demonstrated that exposure to early life stress (ELS) can alter the expression of Nr3c1, which is sustained by changes in the methylation status of its promoter. Such mechanism may contribute to the long lasting consequences of ELS, which may eventually lead to the development of mood disorders (Smart et al., 2015).

Here, on the bases of these observation, we evaluated the functional activity of the HPA axis in our animal model of depression, by focusing on DNA methylation mechanism. Furthermore, we assessed the possible role of the pharmacological intervention with lurasidone in modulating the epigenetic alterations induced by stress exposure.

With these premises, we aim to assess whether the methylation status of the gene encoding for the glucocorticoid receptor Nr3c1 as well as Gadd45β and the Sgk1, two GR responsive genes, could be influenced by chronic stress exposure and by the pharmacological treatment and how

the methylation state may affect genes expression. Moreover, we evaluated the long-lasting effect of stress exposure by measuring the methylation status of these genes after a period of recovery from both chronic stress and the pharmacological treatment.

In particular, we investigated the methylation status on the promotor region of the Nr3c1 gene by describing four CGs methylated in this position. With respect to the two GR responsive genes considered, we analyzed the methylation status of the CGs specifically located on the glucocorticoid responsive element. Specifically, for Gadd45β, based on literature data, we identified on the DNA sequence the possible GRE and we construct the assay in this specific sub region, thus measuring the five CGs herein. For Sgk1, Itani and colleagues in 2002, demonstrated the presence of a GRE on its DNA sequence (Itani et al., 2002) and in the following paragraphs I described the effect of methylation of the two CGs present specifically in this region of Sgk1 DNA (for detail see table in the "materials and methods" section).

1.16.1 Results

1.16.1.1 Modulation of Nrc1, Gadd45β mRNA and Sgk1 gene expression in the prefrontal cortex of chronically stressed rats treated with lurasidone

In order to establish the effect of chronic stress and lurasidone treatment on HPA axis activity, we analysed the mRNA levels of *Nr3c1* and of two GR responsive genes, namely *Gadd45β* and *Sgk1*.

The expression level of the gene encoding for the glucocorticoid receptor was not affected by stress exposure ($F_{1\text{-}36}$: 2.777), by the pharmacological treatment ($F_{1\text{-}36}$: 0.155, $p>0.05$), and by the stressXtreatment interaction ($F_{1\text{-}36}$: 0.834), as revealed by the two-way ANOVA results (fig. 23A). However, despite the fact that *Nr3c1* was not significantly altered in our experimental condition, we investigate the possible modulation of two downstream target of the glucocorticoid receptor signalling, *Gadd45β* and *Sgk1.*

As shown in figure 23B, we found a significant effect of stress ($F_{1\text{-}37}$: 4.840, $p<0.05$) as well as of the treatment ($F_{1\text{-}37}$: 6.971, $p<0.05$) on *Gadd45β* expression. Indeed, 7 weeks of chronic mild stress produced a significant reduction of *Gadd45β* mRNA levels (-18%, $p<0,01$ vs no stress/VEH) compared to non-stressed rats. On the contrary, prolonged lurasidone treatment was able to normalize the CMS-induce downregulation of *Gadd45β* (+40%, $p<0.05$ vs CMS/VEH).

Conversely, as shown by the two-way ANOVA results, *Sgk1* gene expression was not significantly modulated by stress exposure ($F_{1\text{-}40}$: 1.318, $p>0.05$), by the pharmacological treatment ($F_{1\text{-}40}$: 0.362, $p>0.05$) with no significant stressXtreatment interaction (*Sgk1*: $F_{1\text{-}40}$: 0.683, $p>0.05$) (fig. 23C).

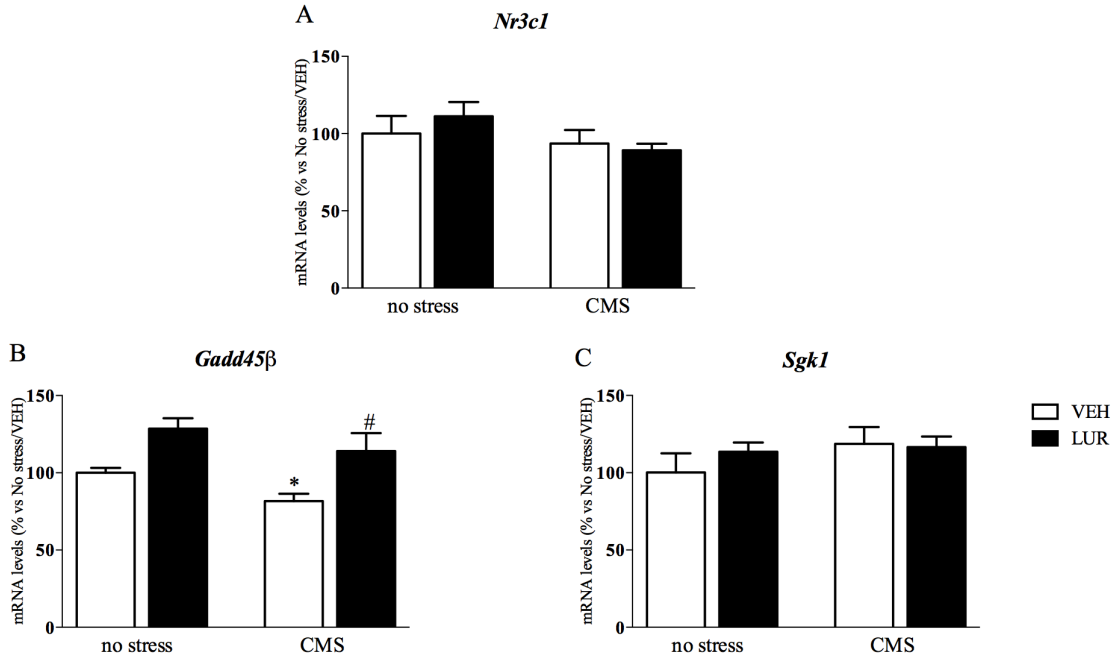

Fig. 23: Analysis of Nr3c1, Gadd45β and Sgk1 mRNA levels in the prefrontal cortex of chronically stressed rats: modulation by chronic lurasidone (LUR) treatment. The data, expressed as a percentage of no stress/VEH animals (set at 100%), are the mean ± SEM of at least six independent determinations. *p<0.05 vs no stress/VEH; # p<0.05 vs CMS/VEH (Two-way ANOVA with PLSD).

1.16.1.2 Modulation of Nr3c1, Gadd45β and Sgk1 DNA methylation levels in chronically stressed rats treated with lurasidone

The results obtained at transcription levels, in particular the significant downregulation of Gadd45β expression induced by chronic stress exposure and the complete normalization due to the prolonged lurasidone administration, suggested us to investigated DNA methylation mechanism, by which the gene may be regulated by both stress and pharmacological treatment. Hence, we assayed the level of Nr3c1, Gadd45β and Sgk1 DNA methylation status in the prefrontal cortex of chronically stressed rats treated with lurasidone.

As shown in figure 24A, the methylation status of Nr3c1 promotor region remained almost stable in all the experimental group, without any statistically significant change (no stress/VEH: 6.3% ± 1.1; no stress/LUR: 5.1%± 1.1; CMS/VEH: 7.1% ± 1.6; CMS/LUR: 5.5% ± 0.9).

Regarding Gadd45β, we assessed the DNA methylation percentage as the mean of five CGs present in the GRE. Interestingly, this region was more methylated in chronically stressed rats (13.39% ± 0.7, $p<0.05$ vs no stress/VEH) as compared to non-stressed group (10.70% ± 0.7) (fig. 24B), a data that is in agreement with the reduction observed for Gadd45β mRNA levels expression in the same group, as described above (see fig. 23B). Interestingly, prolonged lurasidone treatment produced a statistically significant decrease of the methylation percentage in chronically stressed rats (11.31% ± 1, $p<0.05$ vs CMS/VEH), an effect that may contribute to restore Gadd45β mRNA levels in CMS/LUR group (see fig. 23B), with an expression similar to the non-stressed group.

We also analyzed the methylation status of the two CGs present in the GRE sequence on Sgk1 DNA. However, we didn't find any statistically significant change in non-stressed groups (no stress/VEH: 4.2% ± 0.2; no stress/LUR: 4.4± 0.2) as well as in stressed animals (CMS/VEH: 3.9 ± 0.2; CMS/LUR: 4.1 ± 0.2) (fig. 24C).

Moreover, the different grade of methylation of these targets, and in particular the higher % of methylation of the Gadd45β, suggests that its modulation may have more functional consequences in response to environmental stimuli, as compared to the other genes investigated.

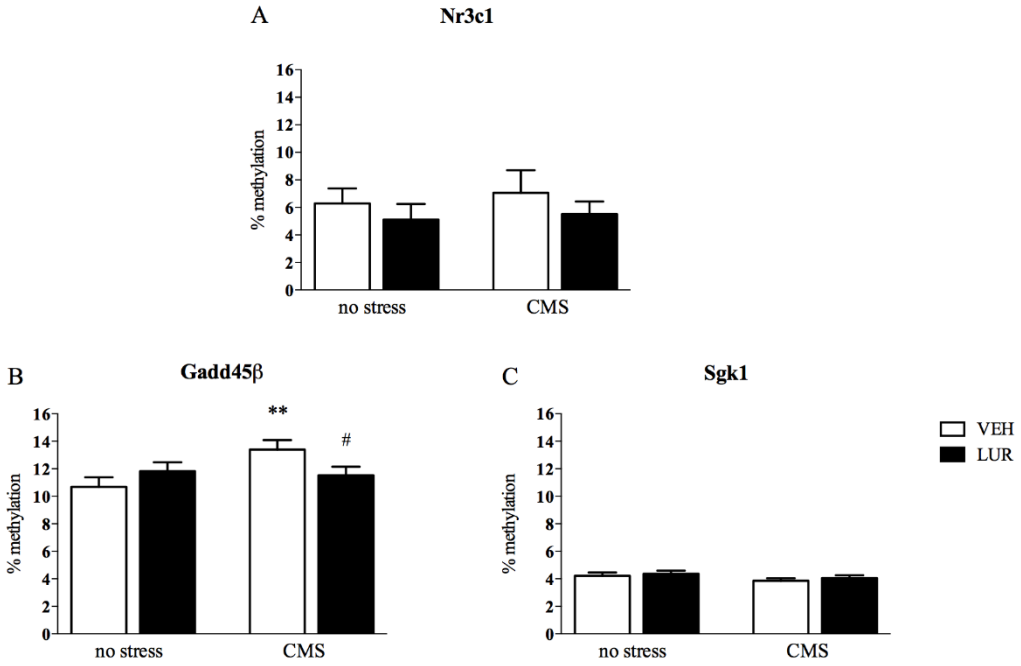

*Fig. 24: Analysis of Nr3c1, Gadd45*β *and Sgk1 DNA methylation in the prefrontal cortex of chronically stressed rats (CMS): modulation by chronic lurasidone (LUR) treatment*. The data, expressed as methylation percentage, are the mean ± SEM of the position analysed of at least 10 independent determinations. **p<0.05 vs no stress/VEH; # p<0.05 vs CMS/VEH (Two-way ANOVA with PLSD).

1.16.1.3 Analysis of Nrc1, Gadd45β mRNA and Sgk1 gene expression in the prefrontal cortex of chronically stressed rats treated after a period of recovery: modulation by chronic lurasidone (LUR) treatment

Next, we decided to investigate if the HPA axis function could be altered by CMS exposure following a period of recovery. Indeed, while it is known that its activity is compromised by chronic stress exposure, little is known about the persistent consequences of prolonged negative adversities on the axis activity.

Hence we investigated the mRNA levels of *Nr3c1*, *Gadd45β* and *Sgk1* mRNA levels in chronically stressed rats (treated with vehicle or lurasidone) after 4 weeks of recovery at the end of stress exposure.

Nr3c1 gene expression, as revealed by the two-way ANOVA analysis, was not significantly modulated after the washout by the stress+washout (F_{1-37}: 0.43, $p>0.05$), by the pharmacological treatment+washout (F_{1-37}: 1.237, $p>0.05$) and there was no interaction between the two variables (F_{1-37}: 1.193, $p>0.05$) (fig. 25A). These data are in line with the results obtained immediately at the end of chronic stress procedure (fig. 23A), thus corroborating the fact that nor stress exposure, neither the lurasidone administration, influenced the expression levels of the glucocorticoid receptor in our experimental condition.

Regarding *Gadd45β*, the analysis of the two-way ANOVA showed a significant effect of stress+washout (F_{1-40}: 8.968, $p<0.01$) and of the treatment+washout (F_{1-40}: 4.293, $p<0.05$) without a significant interaction between the two conditions (F_{1-40}: 2.141, $p>0.05$). Accordingly, in stressed rats exposed to a period of washout, independently from the pre-treatment, we found a significant decrease of *Gadd45β* mRNA levels (-36%, $p<0.01$ vs no stress/washout), an effect that was also observed in non-stressed rats treated with lurasidone (-28%, $p<0.05$ vs no stress/washout) (fig. 25B). This finding suggests that stress exposure has long-lasting and detrimental effect on *Gadd45β* expression.

On the contrary, with respect to *Sgk1*, as confirmed by the two-way ANOVA analysis, we did not observe any statistically significant effect of the stress+washout (F_{1-40}: 1.318, $p>0.05$), of the pharmacological treatment+washout (Sgk1: F_{1-40}: 0.362, $p>0.05$) and of the interaction (Sgk1: F_{1-40}: 0.683, $p>0.05$) (fig. 25C).

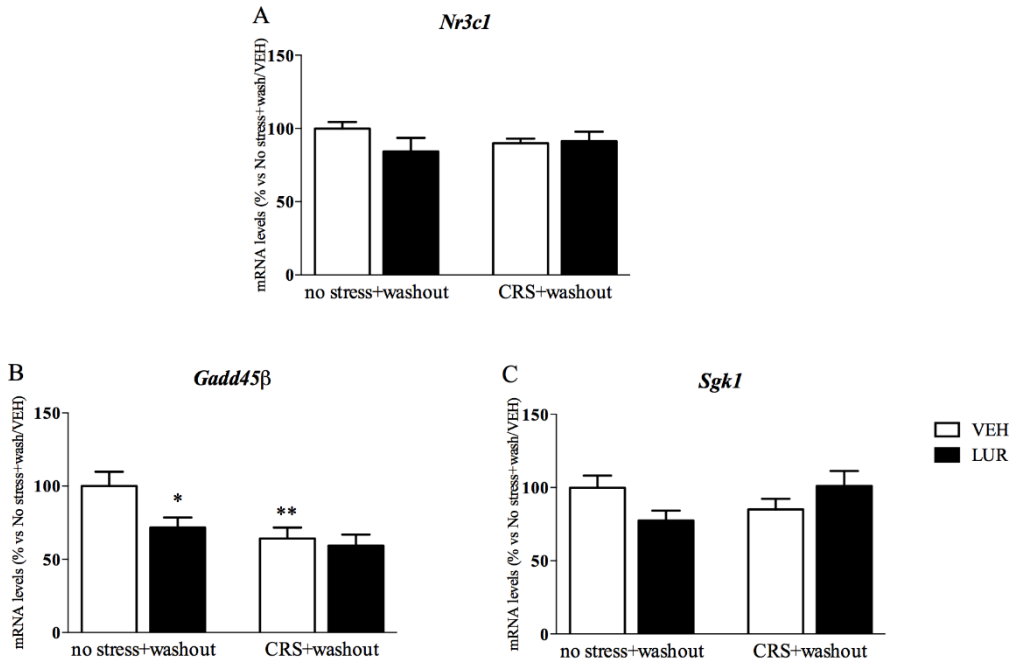

Fig. 25: Analysis of Nr3c1, Gadd45β and Sgk1 mRNA levels in the prefrontal cortex of chronically restraint stressed rats (CRS) after 3 weeks of recovery (washout): modulation by chronic lurasidone (LUR) treatment. The data, expressed as a percentage of no stress+washout/VEH animals (set at 100%), are the mean ± SEM of at least nine independent determinations. *p<0.05 vs no stress+washout/VEH; # p<0.05 vs CRS+washout/VEH (Two-way ANOVA with PLSD).

1.16.1.4 Analysis of Nr3c1, Gadd45β and Sgk1 DNA methylation levels of chronically stressed rats and exposed to a period of recovery: modulation by chronic lurasidone (LUR) treatment

In order to evaluate whether the long-lasting effect of chronic stress exposure were sustained by epigenetic mechanisms, we investigated the DNA methylation levels of Nr3c1, Gadd45β and Sgk1 after three weeks of recovery from both the stress procedure and the pharmacological treatment.

After three weeks of recovery, Nr3c1 promoter region methylation status was not affected by CRS exposure and/or lurasidone treatment (no stress+washout/VEH: 3.9% ± 0.2, $p>0.05$; no stress+washout /LUR: 3.9± 0.4, $p>0.05$; CRS+washout /VEH: 3.9 ± 0.5, $p>0.05$; CRS+washout /LUR: 5.7 ± 1.1, $p>0.05$) (fig. 26A).

On the contrary, as shown in figure 26B, Gadd45β was significantly more methylated after the three weeks of stress washout (12.64± 0.5, $p<0.05$ vs no stress+washout/VEH) as compared to non-stressed rats (10.54 ± 0.5), in line with the decreased of Gadd45β mRNA levels (fig. 25B), suggesting that this epigenetic mechanism may contribute to the persistent changes observed on this GR responsive gene, even after a period of washout. However, lurasidone treatment was not able to counteract this long-lasting modulation induced by CRS.

Similarly, Sgk1 methylation levels were significantly increased after the recovery period from CRS (4.4 ± 0.2 vs no stress+washout/VEH), as compared to no stress+washout group (3.8 ± 0.2) (fig. 26C), even if the *Sgk1* mRNA levels were not significantly modulated in our experimental conditions (fig. 25C).

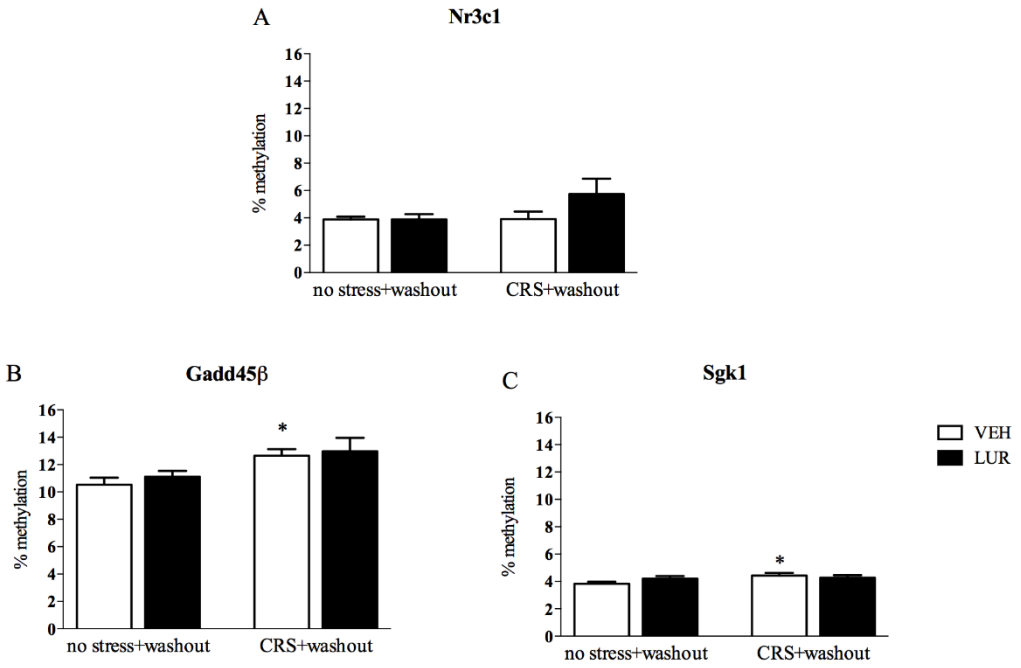

Fig. 26: Analysis of Nr3c1, Gadd45β and Sgk1 DNA methylation in the prefrontal cortex of chronically restraint stressed rats (CRS) after 3 weeks of recovery (washout): modulation by chronic lurasidone (LUR) treatment. The data, expressed as methylation percentage, are the mean ± SEM of the position analysed of at least 10 independent determinations. *p<0.05 vs no stress+washout/VEH (Two-way ANOVA with PLSD).

1.16.2 Discussion

In this section of our study, we provide support to the evidence that chronic stress exposure has a direct impact on the DNA methylation and that the pharmacological treatment with lurasidone may modulate these epigenetics alterations in rat prefrontal cortex. Furthermore, we found that these stress-induced modifications were still present following a period of recovery from stress and in our experimental conditions the drug administration was not protective.

Recent evidence indicates that epigenetic mechanisms, including histone modifications and DNA methylation, are involved in the pathophysiology of depression and in the action of antidepressants (Krishnan and Nestler, 2008) and that such mechanisms may affect several pathways leading to depression-like behaviors in animal models (Massart et al., 2012). In particular, cytosine methylation within the CpG islands in critical regulatory regions alters genes transcription, without changing their sequences, thus having important functional consequences for the regulation of the affected genes (Farrell and O'Keane, 2016). Moreover, the dynamic regulation of DNA cytosine methylation extends beyond the developmental period, being maintained in adult brain, where its alteration can contribute to psychiatric disorders (Moore et al., 2013).

In the field of epigenetics and MDD, the majority of the studies associated early life adversities with long-lasting alterations of DNA methylation of candidate genes for depressive disorders (Weaver et al., 2004a;Murgatroyd et al., 2009), notably involved in the regulation of the HPA axis activity (Weaver et al., 2004b). Here, we assessed the impact of chronic stress exposure ad adulthood, and its possible long-lasting consequences, on the HPA activity, by evaluating the involvement of epigenetic mechanisms, in particular DNA methylation, in the dysregulation of the HPA axis. Moreover, we also examined the contribution of the pharmacological treatment with lurasidone in counteracting molecular abnormalities related with the glucocorticoid receptor signaling.

In our experimental paradigm, nor the *Nr3c1* gene expression, neither its methylation were perturbed by 7 weeks of chronic mild stress or by lurasidone administration and this status did not change after a period of recovery from both stress and the pharmacological treatment.

Despite we did not find any alteration on the glucocorticoid receptor, its epigenetic modification, as link among the HPA axis functionality and stress related disorders, has been widely analyzed. Indeed, it has been demonstrated that early life adversities were able to modify the methylation status of the Nr3c1, thus leading to long-lasting consequences later in life including the development of mood disorders (Smart et al., 2015). Moreover, depressed patients have high levels of Nr3c1 methylation (Farrell et al., 2018) and Massart and colleagues found altered epigenetic regulators expression in GR-I mice, a genetic model of depression (Massart

et al., 2012). Among the downstream targets of GR, we found that 7 weeks of chronic mild stress decreased Gadd45β mRNA levels, indicating that regulatory mechanisms involved in the transcription of genes, may be affected by stress exposure, thus altering their expression. These results were in line with the findings of Grassi and colleagues who demonstrated that chronic mild unpredictable stress produced a significant down-regulation of the demethylase *Gadd45β* mRNA levels in the prefrontal cortex of mice (Grassi et al., 2017).

Interestingly, this transcriptional change was sustained by epigenetic modification, with an overall increase of the methylation percentage at the GRE of Gadd45β, due to stress exposure. We hypothesize that chronic stress induced-methylation of these CGs could prevent the binding of transcriptional regulatory proteins at the GRE site, thus altering the down-stream activity mediated by the HPA axis functioning. Moreover, after three weeks of recovery, we found a persistent Gadd45β methylation status due to stress pre-exposure, suggesting an enduring effect of chronic stress exposure. Interestingly chronic lurasidone treatment was able to normalize not only the CMS induced reduction of Gadd45β expression, but also the changes of the DNA methylation status due to chronic stress. These results suggest that lurasidone may exert a protective effect toward stress, also by interfering with the epigenetic alterations produced by the adverse experience. The finding that chronic lurasidone treatment was able to revert the DNA methylation changes when chronically administered during the pathological condition represents an important observation in the field of the "epigenetically targeted interventions", to alleviate adverse phenotype (Szyf et al., 2016). Conversely, we did not observe the beneficial effect of the drug in the post-treatment period. Indeed, after the cessation of chronic lurasidone administration, the long-lasting stress-induced changes in expression and methylation of Gadd45β were not normalized by the drug.

In line with the CMS-modulation of Gadd45β, prolonged stress had persistent consequences also on Sgk1 methylation in the CGs of the GRE, independently from lurasidone administration, even if the degree of change was minor than the one observed for the Gadd45 β. Accordingly, Sgk1 has been identified as DNA methylation biomarker of MDD since its methylation status as been found to be altered in the peripheral blood of depressant patients (Numata et al., 2015). Our results indicated that chronic stress affected and compromised mainly Gadd45β expression, acting at both transcriptional and epigenetic level. These effects persisted even after the recovery period, pointing out that Gadd45β may represent a stable stress-induced molecular scar in the rat prefrontal cortex. Moreover, the observation that the methylation status of this GR responsive gene was completely reverted by the lurasidone treatment, indicated that Gadd45β may be a candidate marker for pharmacological treatment of stress-related disorders.

Furthermore, seen the long-lasting consequence of stress on the methylation profile of both Gadd45β and Sgk1, whereas no endurable effect of the drug, our results suggested the need to extend the drug administration after the cessation of the stress protocol in order to avoid the rebound mechanisms driven by the suspension of lurasidone during the recovery. Thus, it may be proposed that the continuation of the treatment, even after the remission, could have a beneficial effect in preventing further relapses as well as in acting on the persistent epigenetic alterations connected with chronic stress, that may represent scars of vulnerability to the pathology recurrence.

In conclusion, our data highlight that chronic stress exposure results in persistent changes in DNA methylation in specific genes related with glucocorticoids signalling and that lurasidone acts as a modifier of such mechanisms, suggesting its potential as modulator of the HPA axis that is compromised in different psychiatric disorders (de Kloet et al., 2005;Pariante and Lightman, 2008). Moreover, these epigenetic alterations may be connected with the behavioural deficits we observed in the chronic mild stress animal model of depression, and the rescue of these symptoms by lurasidone treatment (Calabrese et al., manuscript in preparation- chapter 4.2) may be linked with the drug possible properties as "epigenetic modulators".

1.17 Long-term outcomes of chronic restraint stress and lurasidone treatment on brain plasticity and responsiveness to an acute challenge

Unpublished data

Depression has been largely characterized as a recurrent disorder, with approximately 50% of patients that experience relapse (Keller, 2003). Indeed, while it is expected that antidepressant or other psychotropic drugs used in the treatment of depression may prevent relapse, limited knowledge exists 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 have an adverse impact on the long-term course of the disorder and it may increase the response to subsequent stressors. Indeed, it is possible that not all the systems impaired by stress are restored during the remission, thus leaving 'scars' of vulnerability that may facilitate the relapse to the pathology.

Furthermore, even if at preclinical level chronic stress exposure at adulthood has been widely described as environmental factor able to induce depressive phenotype in rodents, limited information is available on the long-lasting impact of stress as well as on the mechanisms that may promote or prevent relapse. Actually, the effect of stress may not only have persistent consequences, but may physiologically be recovered through the activation of dynamic processes that help the brain to achieve successful adaptation (McDowell et al., 2015).

Hence, the main purpose of this experiment was to investigate if and how stress-induced changes 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 lurasidone, to better understand if pharmacological intervention may lead to a more complete normalization of the molecular alterations induced by stress, which may account for a reduced susceptibility to subsequent negative events.

To address these objectives, we exposed adult rats to four weeks of chronic restraint stress and we left them to recover for the subsequent three weeks, to identify the functional abnormalities that may render them more 'vulnerable' under a challenging precipitating condition in the rest period. Hence, after the recovery the animals were presented to an acute immobilization stress in order to establish differences in molecular responsiveness to the stressful event, in animals that were originally exposed to the CRS procedure and had received vehicle or lurasidone treatment. We focus our analyses on the prefrontal cortex and dorsal hippocampus, brain region primary involved in the stress response and in the integration of information for past with present stimuli (Fuster et al., 2000).

1.17.1 Results

1.17.1.1 Analysis of Arc mRNA levels following a period of recovery from chronic restraint stress and prolonged lurasidone treatment

First, we investigated the expression level of the activity-dependent gene *Arc,* a key element of neuronal activation associated with ongoing behavioral activity, to assess neuronal responsiveness after three weeks of recovery from chronic restraint stress and lurasidone treatment. In the prefrontal cortex (fig. 27A), we found a significant effect of the stress (F_{1-39}=6.828, p<0.05) and of the treatment (F_{1-39}=7.066, p<0.05). In particular, after the recovery period from stress, the expression of the IEG was significantly down-regulated by CRS alone (-29%, p<0.01 vs no stress+washout/VEH) in comparison to non-stressed rats. Lurasidone treatment significant decreased *Arc* mRNA levels (-29%, p<0.01 vs no stress+washout/VEH) in non-stress rats while no further differences were found in animals exposed to CRS.
Conversely, in the dorsal hippocampus we did not observe any long-lasting modulation on *Arc* expression, nor by the prolonged stress exposure, neither by the pharmacological treatment (fig. 27B).
These results indicated that *Arc* expression was persistently decreased by stress in the prefrontal cortex and that the concomitant treatment with lurasidone was not able to counteract the effect of the CRS, probably caused by rebound mechanisms set in motion by drug withdrawal, seen the downregulation induced also by the drug in non-stressed animals.

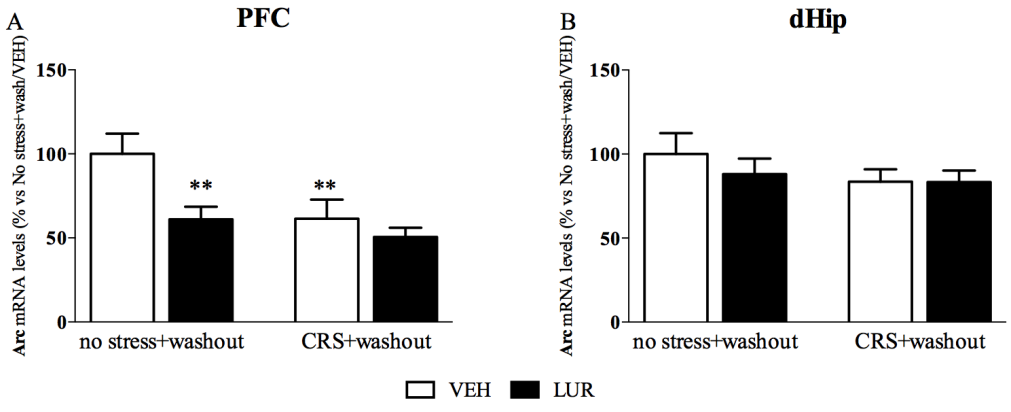

Fig. 27: Analysis of Arc mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of chronically stressed rats (CRS), treated with lurasidone (LUR), after 3 weeks of recovery (washout). The data, expressed as percentage of no stress+washout, are the mean ± SEM of at least 9 independent determinations. **p<0.01 vs no stress+washout/VEH (Two-way ANOVA with PLSD).

1.17.1.2 Analysis of total Bdnf and Bdnf long 3'UTR mRNA levels following a period of recovery from chronic restraint stress and prolonged lurasidone treatment

On the basis of our previous studies demonstrating that lurasidone is effective in up-regulating the expression of the neurotrophin Bdnf and can restore its alterations as a consequence of the exposure to stressful experiences (Fumagalli et al., 2012;Luoni et al., 2015), we investigated the long-lasting effect of chronic stress exposure and of lurasidone administration on the expression of both total *Bdnf* and *Bdnf* long 3'UTR. In the prefrontal cortex, as shown in figure 28A, two-way ANOVA revealed a significant effect of the stress ($F_{1\text{-}39}$=11.071, $p<0.05$) on the total form of the neurotrophin. Indeed, we found that animals exposed to CRS showed a decrease of total *Bdnf* mRNA levels after 3 weeks wash-out (-35%, $p<0.01$ vs no stress+washout/VEH), even when the animals were chronically treated with lurasidone during the stress exposure. On the contrary, *Bdnf* long 3'UTR pool of transcripts were not modulated in any experimental groups (fig. 28B).

In the dorsal hippocampus, we did not find any significant change of total *Bdnf* and *Bdnf* long 3'UTR mRNA levels after the wash-out from stress or lurasidone treatment (figure 28 C-D).

These results suggest that chronic restraint stress appear to have a stable detrimental effect on the expression of the total form of the neurotrophin mainly in the prefrontal cortex, whereas the dorsal hippocampus is less vulnerable to stress pre-exposure in term of *Bdnf* expression. Furthermore, prolonged lurasidone administration during stress exposure was not able to prevent the downregulation of *Bdnf* expression in CRS rats following a period of washout (recovery).

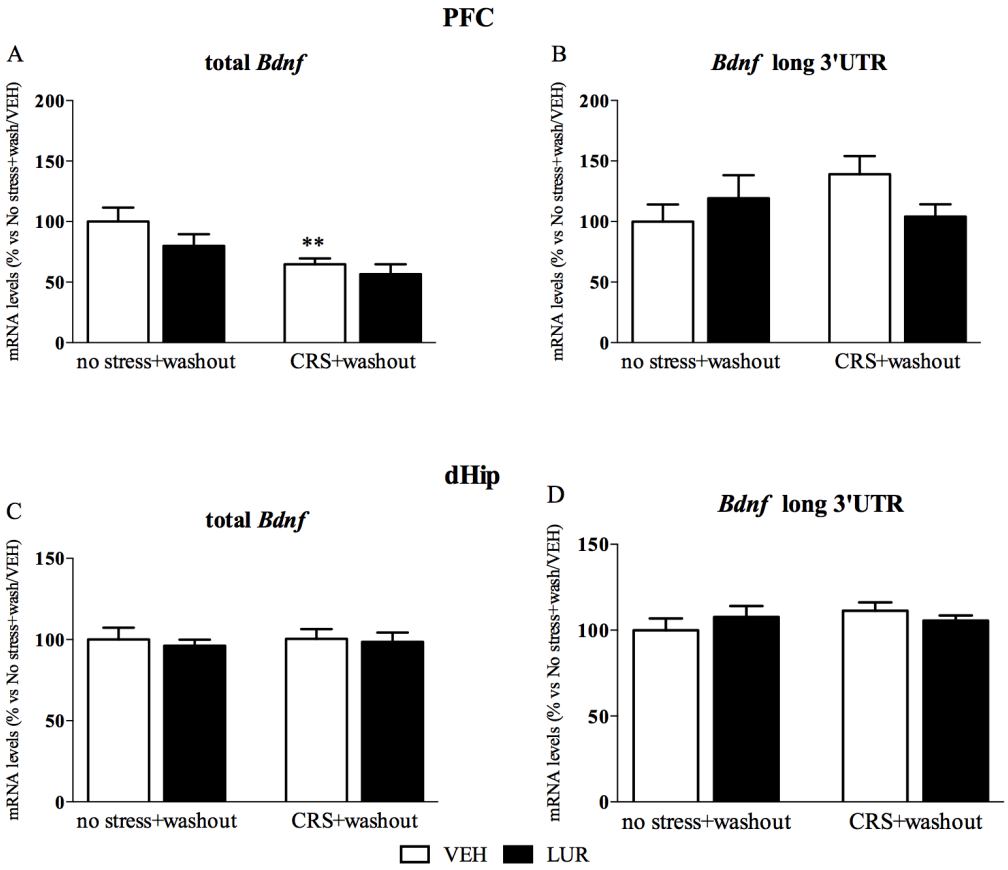

Fig. 28: Analysis of total Bdnf and Bdnf long 3'UTR mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of chronically stressed rats (CRS), treated with lurasidone (LUR), after 3 weeks of recovery (washout). The data, expressed as percentage of no stress+washout, are the mean ± SEM of at least 9 independent determinations. **p<0.01 vs no stress+washout/VEH (Two-way ANOVA with PLSD).

1.17.1.3 Analysis Gadd45β and Sgk1 mRNA levels following a period of recovery from chronic restraint stress and prolonged lurasidone treatment

In the prefrontal cortex, as describes above (see paragraph 4.3.1, figure 23), *Gadd45β* expression was significantly downregulated by the chronic restrain stress protocol and by lurasidone in non-stressed rats and in CRS group the drug did not alter the stress effect. Conversely, nor the stress, neither the treatment had persistent effect on *Sgk1* mRNA levels.
In the dorsal hippocampus, (fig. 29A), similarly to what observed in PFC, two-way ANOVA revealed a significant effect of the stress ($F_{1\text{-}40}$=6.665, p<0.05) on *Gadd45β*. Indeed, we found that animals exposed to CRS showed a decrease of *Gadd45β* levels after 3 weeks of wash-out (-24%, p<0.01 vs no stress+washout/VEH), even when the animals were chronically treated with lurasidone during the stress exposure. Furthermore, lurasidone induced a significant persistent downregulation also in non-stressed rats (-18 %, p<0.05 vs no stress+washout/VEH). On the contrary, in dHip, *Sgk1* was not modulated in any experimental groups (fig. 29B).
These results suggest that chronic restraint stress have a stable detrimental effect on *Gadd45β* both in the prefrontal cortex and in dorsal hippocampus and that lurasidone was not able to counteract the stress effect, whereas *Sgk1* was not affected by the experimental conditions.

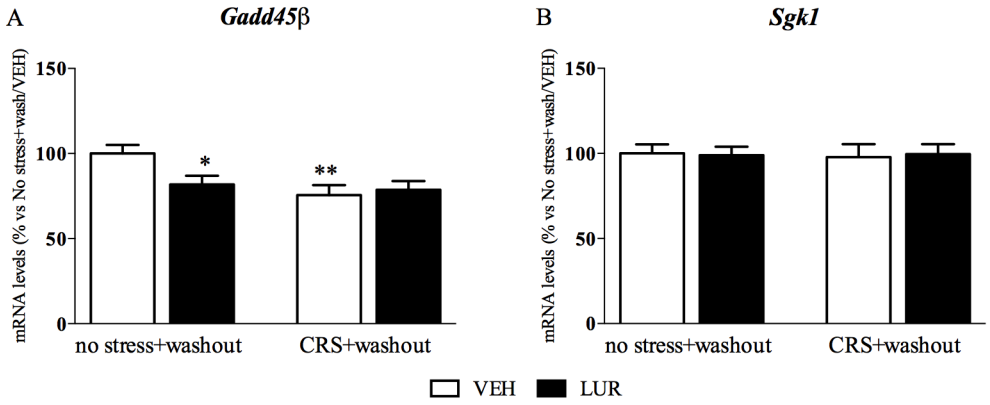

Fig. 29: Analysis of Gadd45β and Sgk1 mRNA levels in the dorsal hippocampus (dHip) of chronically stressed rats (CRS), treated with lurasidone (LUR), after 3 weeks of recovery (washout). The data, expressed as percentage of no stress+washout, are the mean ± SEM of at least 9 independent determinations. * p<0.05, **p<0.01 vs no stress+washout/VEH (Two-way ANOVA with PLSD).

1.17.1.4 Analysis of the effects produced by an acute restraint stress on Arc mRNA levels after a period of recovery from chronic restraint stress and prolonged lurasidone treatment

To evaluate if stress exposure and/or lurasidone treatment may alter the ability to respond to a challenging environmental stimulus, we next examined the effects of an acute restraint stress on the IEG *Arc* at the end of the 3 weeks of washout.

In prefrontal cortex (fig. 30A), we found that, independently from the CMS and the pharmacological treatment, the acute stress (restraint stress: $F_{1\text{-}79}$=91.453, p<0.001, three-way ANOVA analysis), produced an increase of *Arc* in all groups (no stress+wash/VEH/restraint:+ 74 %, p<0.001 vs no stress+wash/VEH/naive; no stress+wash/LUR/restraint: +170%, p<0.001 vs no stress+wash/LUR/ naive; CRS+wash /VEH/restraint: +138% , p<0.001 vs CRS+wash /VEH/naive; CRS+wash /LUR/restraint: +163%, p<0.001 vs CRS+wash /LUR/naive) in comparison with naive counterparts, suggesting that after the washout period, nor the treatment neither the CRS have long-lasting impact on the response to the acute challenge in this brain region.

In dorsal hippocampus (fig. 30B), we found a significant effect of the acute stress ($F_{1\text{-}79}$=25.035, p<0.001) and of the treatment ($F_{1\text{-}79}$=4.380, p<0.001). Indeed, exposure to the acute restraint stress increased *Arc* gene expression in non-stressed rats (+33 %, p<0.001 vs no stress+wash/VEH/Naive) as well as in CRS rats treated with vehicle (+54%, p<0,001 vs CRS+wash /VEH/Naive) or with lurasidone (+43%, p<0,001 vs CRS+wash /LUR/Naive), but not in non-stressed rats treated with the drug (+15%, p>0,05 vs no stress+wash /LUR/Naive).

All in all, in both brain regions considered, acute stress exposure after a period of washout from chronic stress, produced a neuronal activation similar to control rats, without any significant effect of lurasidone treatment.

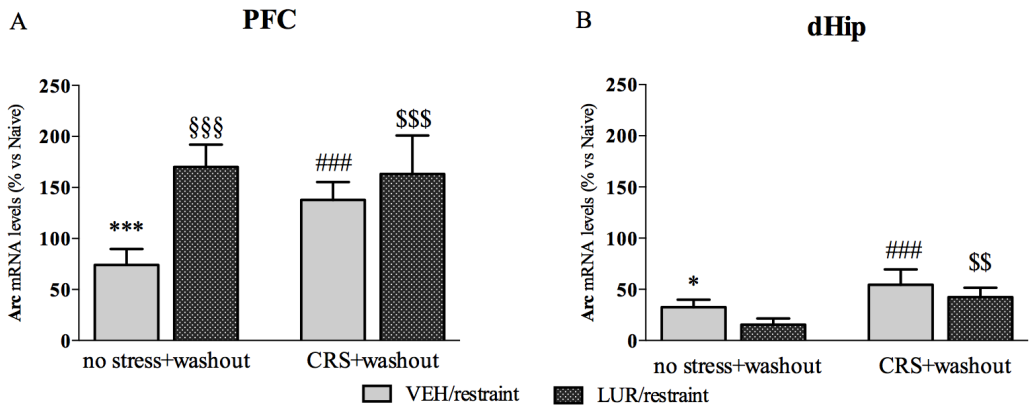

Fig. 30: Analysis of Arc mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of chronically stressed rats (CRS), treated with lurasidone (LUR), and exposed to an acute challenge (restraint) after 3 weeks of recovery (washout). The data, expressed as percent change of animals exposed to the acute stress vs. their sham counterpart, are the mean ± SEM of at least 9 independent determinations. *p<0.05, ***p<0.001 vs no stress+washout/VEH/Naïve; §§§ p<0.001 vs no stress+washout/LUR/Naïve; ### p<0.001 vs CRS+washout/VEH/Naïve; $$ p<0.01, $$$ p<0.001 vs CRS+washout/LUR/Naïve (Three-way ANOVA with PLSD).

1.17.1.5 Analysis of the effects produced by an acute restraint stress on total Bdnf and Bdnf long 3'UTR mRNA levels after a period of recovery from chronic restraint stress and prolonged lurasidone treatment

We also assessed the effects of the acute challenge following the 3 weeks of recovery on total *Bdnf* and *Bdnf* long 3'UTR expression.

In the prefrontal cortex, we found a significant increase of the total form of the neurotrophin (fig. 31A) due to the acute stress in control rats previously treated with lurasidone (+32%, $p<0.05$ vs No stress+wash /LUR/Naive), as compared to the naïve counterpart, whereas no modification was observed in rats pre-exposed to CRS. The pool of *Bdnf* transcripts with the long 3'UTR was modulated by the acute stress ($F_{1-79}=40.846$, $p<0.001$ three-way ANOVA results) with a significant treatmentXrestraint interaction ($F_{1-79}=4.140$, $p<0.05$). Indeed, long 3'UTR *Bdnf* mRNA levels were significantly up-regulated in non-stressed rats treated with vehicle (+63 % $p<0.001$ vs no stress+wash/VEH/Naïve) and lurasidone (+74 % $p<0.01$ vs no stress+wash/LUR/Naïve) and in chronically stressed rats administered with lurasidone (+124%, $p<0,001$ vs CRS+wash /LUR/Naive), but not in the CRS group treated with vehicle (+34%, $p>0.05$ vs CRS+wash /VEH/Naive). Interestingly, the pharmacological treatment enhanced the response to the challenge, mainly in stressed rats, thus suggesting a potential protecting effect of lurasidone administration in the prefrontal cortex (fig. 31B).

On the contrary, in dorsal hippocampus, we found an overall downregulation of the neurotrophin following the acute challenge. Indeed, total *Bdnf* was regulated by stress exposure ($F_{1-80}=8.001$, $p<0.05$) and its expression was decreased by the acute stress, specifically in CRS rats treated with lurasidone (-20%, $p<0.05$ vs CRS+wash /LUR/Naive) (fig. 31C). Similarly, as shown in figure 31D, the acute restraint stress decreased *Bdnf* long 3'UTR gene expression not only in non-stressed rats treated with Lurasidone (-18%, $p<0.05$ vs no stress+wash/LUR/Naïve) but also in CRS rats treated with vehicle (-15%, $p<0,05$ vs CRS+wash/VEH/Naïve) or with lurasidone (-14%, $p<0,05$ vs CRS+wash /LUR/Naive), as confirmed by three-way ANOVA ($F_{1-80}=16.081$, $p<0.05$).

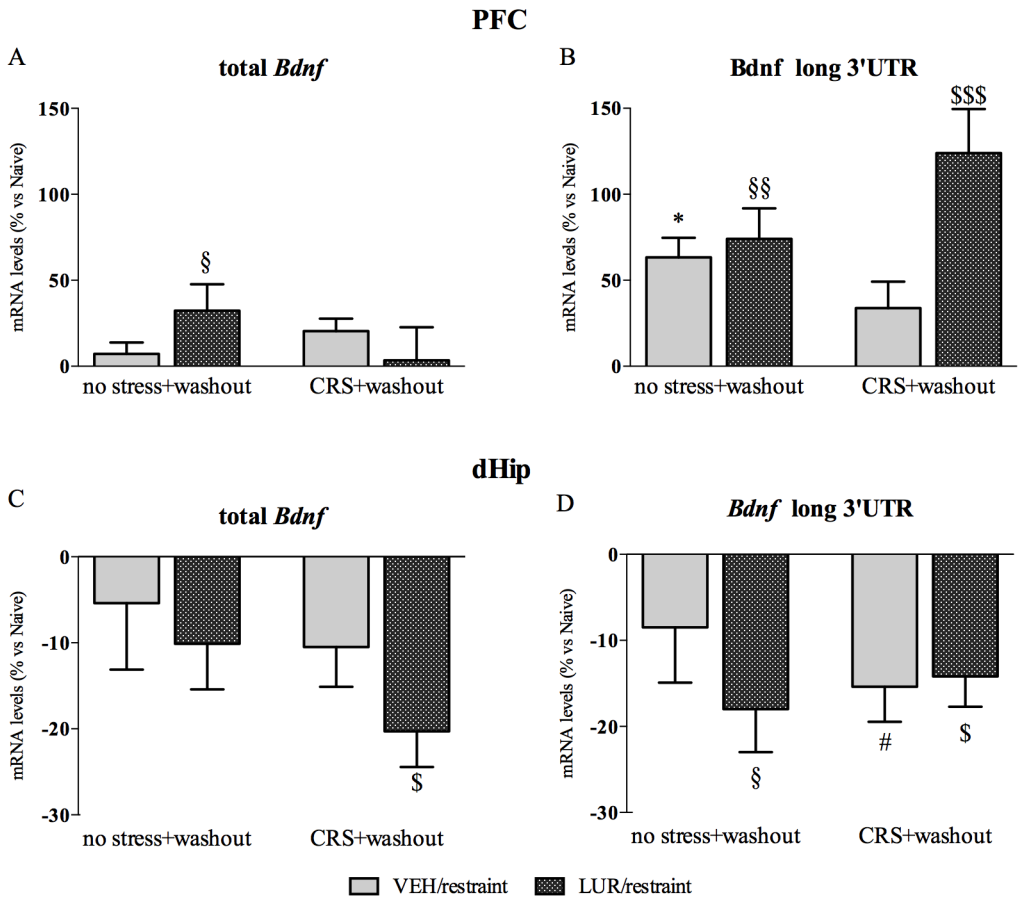

Fig. 31: Analysis of total Bdnf and Bdnf long 3'UTR mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of chronically stressed rats (CRS), treated with lurasidone (LUR), and exposed to an acute challenge (restraint) after 3 weeks of recovery (washout). The data, expressed as percent change of animals exposed to the acute stress vs. their sham counterpart, are the mean ± SEM of at least 9 independent determinations. *p<0.05 vs no stress+washout/VEH/Naïve; § p<0.05, §§ p<0.01 vs no stress+washout/LUR/Naïve; # p<0.05 vs CRS+washout/VEH/Naïve; $ p<0.05, $$$ p<0.001 vs CRS+washout/LUR/Naïve (Three-way ANOVA with PLSD).

1.17.1.6 Analysis of the effects produced by an acute restraint stress on Gadd45β and Sgk1 mRNA levels after a period of recovery from chronic restraint stress and prolonged lurasidone treatment

Next, we decided to investigate the expression levels of two activity-dependent immediate early genes, *Gadd45ß* and *Sgk-1*, which have different implications for the rapid neuronal response to stress, since their transcription is strictly related to the activation of the glucocorticoid receptor signaling.

In the prefrontal cortex, independently from CRS exposure and pharmacological treatment, the acute stress produced a significant increase of *Gadd45β* mRNA levels, as compared to rats under resting conditions (+51 %, $p<0.001$ vs no stress+wash/VEH/Naïve; +110%, $p<0.001$ vs no stress+wash/LUR/Naïve; +55%, $p<0.01$ vs CRS+wash/VEH/Naïve; +74%, $p<0.01$ vs CRS+wash/LUR/Naïve), as confirmed by the acute restraint significant effect ($F_{1-80}=75.196$, $p<0.001$) (fig. 32A).

Similarly, the acute challenge produced a significant up-regulation of *Sgk1* mRNA levels in both non-stressed (VEH: 100%, $p<0.001$ vs no stress+wash/VEH/Naïve; LUR: +245%, $p<0.001$ vs no stress+wash/LUR/Naïve) and chronically restraint stressed animals (VEH: +105%, $p<0.001$ vs CRS+wash/VEH/Naïve; LUR: +75%, $p<0.01$ vs CRS+wash/LUR/Naïve) (fig. 32B), and these effects appear to be larger in no stress rats treated with lurasidone, as confirmed by the three-way ANOVA results (restraint: $F_{1-78}=120.016$, $p<0.001$; stressXrestraint interaction: $F_{1-78}=9.091$, $p<0.01$; stressXtreatmentXrestraint interaction: $F_{1-78}=6.228$, $p<0.05$), suggesting that lurasidone may exert a positive sustained effect on the acute stress-dependent modulation of *Sgk1*, mainly in non-stressed rats, thus indicating that stress was able to limit the lurasidone induced-changes.

When investigating the dorsal hippocampus, we found that the acute challenge up-regulated *Gadd45β* expression in non-stressed rats treated with Lurasidone (+42%, $p<0,001$ vs no stress+wash/LUR/Naïve) as well as in chronically stressed animals treated with vehicle or with lurasidone (+49%, $p<0,001$ vs CRS+wash/VEH/Naïve; +57%, $p<0,001$ vs CRS+wash/LUR/Naïve, respectively), accordingly to the three-way ANOVA ($F_{1-80}=57.724$, $p<0.05$) (fig. 32C).

In line with what observed for *Gadd45β*, we found an effect of the acute stress on *Sgk1 (*$F_{1-80}=149.984$, $p<0.001$), with an up-regulation of its expression in all the experimental groups exposed to the challenge (+129 %, $p<0.001$ vs no stress+wash/VEH/Naïve; +127%, $p<0.001$ vs no stress+wash/LUR/Naïve; +84%, $p<0.01$ vs CRS+wash/VEH/Naïve; +136%, $p<0.01$ vs CRS+wash/LUR/Naïve), as compared to their naïve counterparts (fig. 32D). In summary, these

results suggest that, in this brain region, the up-regulation of these two activity dependent genes, following the acute challenging stress, is not altered by previous chronic stress exposure.

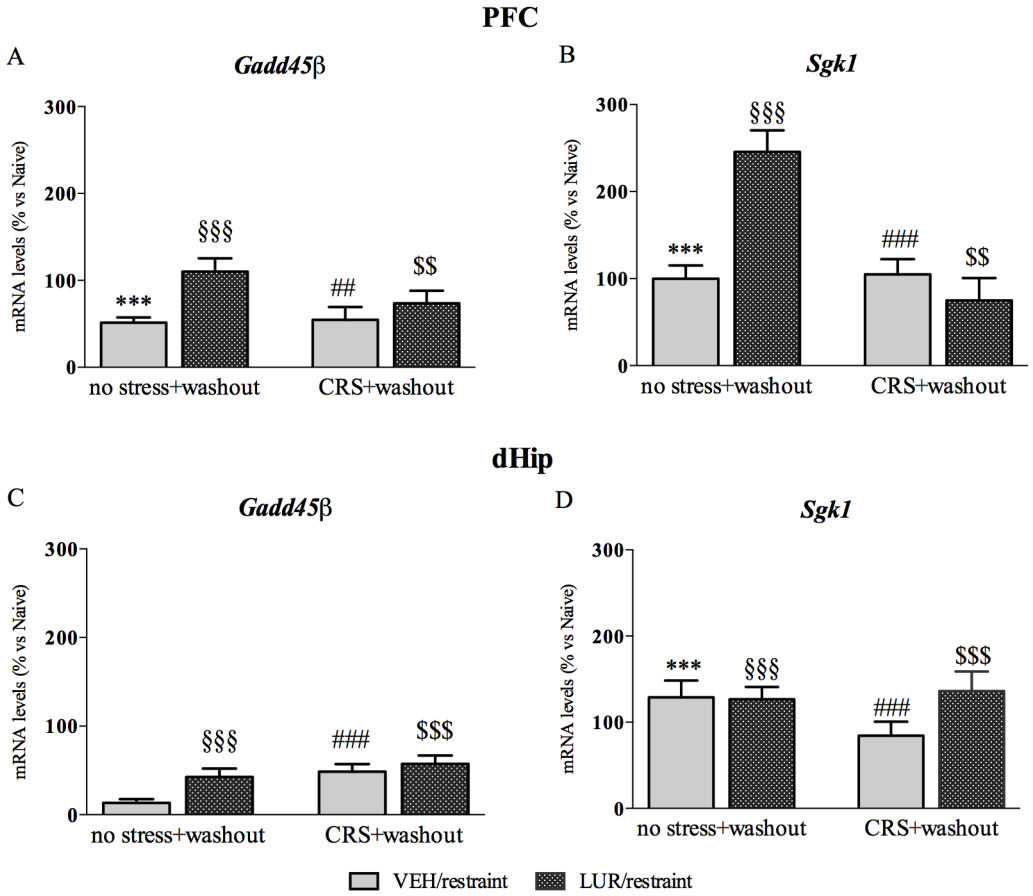

Fig. 32: Analysis of Gadd45β and Sgk-1mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of chronically stressed rats (CRS), treated with lurasidone (LUR), and exposed to an acute challenge (restraint) after 3 weeks of recovery (washout). The data, expressed as percent change of animals exposed to the acute stress vs. their sham counterpart, are the mean ± SEM of at least 9 independent determinations. ***p<0.001 vs no stress+washout/VEH/Naïve; §§§ p<0.001 vs no stress+washout/LUR/Naïve; ## p<0.01, ### p<0.001 vs CRS+washout/VEH/Naïve; $$ p<0.01, $$$ p<0.001 vs CRS+washout/LUR/Naïve (Three-way ANOVA with PLSD).

1.17.2 Discussion

In this work we demonstrated that chronic restraint stress produces protracted molecular changes that may be found also following a period of recovery. Nevertheless, we highlighted the ability of the prefrontal cortex and dorsal hippocampus to display plasticity to deal with challenging condition, in term of neuronal activation and neuroplastic mechanisms, when the animals were exposed to an acute stress after a wash-out period from CRS. However, the concomitant treatment with lurasidone did not modify the response to the acute challenge in our experimental conditions.

Despite the majority of depressed patients may achieve remission following successful pharmacological treatment, there is a high percentage who experience relapse, usually as a consequence of environmental adversities. Furthermore, around 30% of MDD 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 the relapse.

On these bases, we investigated the post-stress period and the ability of the brain the react to a subsequent challenge, in order to identify long-lasting stress-induced changes, and to investigate the ability of lurasidone to improve such alterations.

After three weeks of washout from stress and lurasidone, the activity dependent gene *Arc,* an important target in neuroadaptation (Tzingounis and Nicoll, 2006), was significantly decreased in the prefrontal cortex of stressed rats, an effect that was observed after 11 days of chronic restraint protocol (Ons et al., 2010), suggesting a persistent effect of stress on *Arc* expression.

With regard to neuroplasticity, the detrimental effect of chronic stress on the neurotrophin Bdnf in animal models of depression is well established (Duman and Monteggia, 2006;Calabrese et al., 2009;Calabrese et al., 2014). Here, we demonstrated that chronic restraint stress had a long-lasting, enduring negative effect on the total form *Bdnf* in the prefrontal cortex, which was present even after three weeks of washout, suggesting that the effects produced by stress may persist well beyond the end of the adverse experience. On the contrary, in the dorsal hippocampus, 21 days following the end of the stress we did not observe any changes of both total *Bdnf* and *Bdnf* long 3'UTR pool of transcripts, in line with the findings corroborated by other groups at transcriptional (Lakshminarasimhan and Chattarji, 2012) and translational level (Xu et al., 2004).

Despite the fact that chronic treatment with lurasidone is able to normalize the changes induced by stress on *Bdnf expression* (Fumagalli et al., 2012;Luoni et al., 2015), here we show that, following three weeks of washout from the end of stress or drug administration, lurasidone did not exert enduring protective effects in the PFC of CRS rats, suggesting that the drug was inefficient in preventing the neuroplastic alterations induced by stress. One possible caveat for

these results is that the sudden interruption from lurasidone treatment may generate a sort of 'withdrawal' that has a negative impact on neuroplasticity. Hence, it may be interfered, that the drug could exert long-lasting beneficial effect if not interrupted.

In the prefrontal cortex, after the recovery, both the treatment and stress exposure decreased *Gadd45β* expression with respect to control animals, in line with the downregulation of *Gadd45β* in the PFC that has been detected following the chronic mild unpredictable stress protocol (Grassi et al., 2017). Our result, interestingly, indicated the endurable consequence of 4 weeks of CRS procedure on *Gadd45β* expression.

Furthermore, in this brain region, as suggested above, lurasidone treatment may produce rebound mechanisms probably set in motion by drug withdrawal, since *Arc* and *Gadd45β* mRNA levels were downregulated after 3 weeks of the treatment interruption also in non-stressed animals. Instead, we did not find endurable effects of the stress in the dorsal hippocampus, indicating that during the washout period, some systems may be activated specifically in this brain area to counteract the persistent consequences of negative stressors.

Accordingly, different studies demonstrated that the hippocampus appear to "recover" as weeks pass from the end of the chronic stress (Hoffman et al., 2011;Ortiz et al., 2014;Ortiz et al., 2015;Ortiz et al., 2018) and that the recovery of the depressive like behavior, induced by CMS, was achieved after 4 weeks of washout (Alves et al., 2017).

Next, in order to assess if the physiological improvement of the post-stress wash-out period may influence the response to a subsequent acute challenge, we exposed rats to one hour of acute restraint stress, the same type of stress rats were subjected during the four weeks of CRS, to investigate if the memory of the experienced stressors may impact on brain responsiveness after recovery. Furthermore, we evaluated the possible protective effect of the lurasidone pre-administration.

When we measured *Arc* expression, independently from the stress and the treatment, in both prefrontal cortex and dorsal hippocampus, we observed a rapid and strong upregulation of the IEG, in response to the challenge. On the contrary, the downregulation of *Bdnf* in the post-stress rest period appeared to inhibit PFC activation: indeed, both the total and long 3' UTR *Bdnf* were not enhanced by the acute stress, suggesting an impairment in the neurotrophin-ability to cope with the challenging situation due to the long-lasting detrimental effect of CRS.

Interestingly, chronically stressed rats treated with lurasidone showed enhanced response to the challenge, thus suggesting a potential persistent protecting effect of the drug in the prefrontal cortex. Conversely, the dorsal hippocampus appeared to be impaired by the acute restraint, with in particular *Bdnf* long 3' UTR being down-regulated independently from the stress and treatment, indicating a lasting negative sensitization to the novel stressor.

Finally, the rapid stress neuronal response, in term of the expression of the two activity-dependent immediate early genes, *Gadd45ß* and *Sgk1*, was not prevented by the CRS in both the brain regions, independently from the treatment, indicating that their up-regulation, in response to new stimuli, was not impaired after the recovery. Moreover, the massive increase of *Sgk1* in non-stressed rats treated with lurasidone suggested that the drug can exert a positive sustained effect on the acute stress-dependent modulation of *Sgk1*expression, mainly in PFC.

Taken together, this study indicates that the prefrontal cortex was mainly implicated in the adaptation to stressors, exerting its ability to display plasticity after the post-stress wash-out period, in particular by adequately reacting to a subsequent acute stress exposure in term of the activity-dependent immediate early genes *Arc, Gadd45ß* and *Sgk1*, in line with the notions that PFC is a brain region with high levels of structural and functional plasticity, that permits itself to modify brain functioning by internalizing behavioral experiences (McEwen and Morrison, 2013). In particular, these results suggest that the recovery period allowed the rats to rescue some chronic alterations produced by the prolonged stress exposure, implicating that the rapid neuronal activation had returned to a "previous state", independently from lurasidone. Despite of that, the long-lasting negative effect of the chronic stress on the neurotrophin *Bdnf* interfered with the ability of the PFC to cope with a challenging condition, thus leading to the lack of response, in term of *Bdnf,* following the acute challenge.

In conclusion, we highlight modification, induced by chronic stress, of distinct systems in the two areas considered, and their different adaptations and support during the recovery period. Moreover, these results reveal the complexity of the plastic mechanisms set in motion to cope with challenges, mainly when the system was previously impaired, adding critical new information in the field of better understanding the ways to promote mechanisms of adaptive plasticity.

1.18 Effect of acute stress on the cognitive performance: a role for neuroplastic mechanisms

Unpublished data

The brain is the primary organ that response to stressful stimuli in order to cope with homeostatic challenges. It is well documented that exposure to mild and brief stressors provide beneficial advantages in a short-term, period by activating protective functions or by preparing the organism to react with external demands. Furthermore, when the stress is short, it can have positive effects on memory (Joels et al., 2006) and even be fundamental for good learning (Sandi and Rose, 1994;Sandi et al., 1997). Here, to determine the consequences of an acute stress on memory processes, we tested adult rats in a cognitive task at different time points, in order to have a time-course of the effect of the stress. Specifically, rats were exposed to the novel object recognition test 1 hour, 4 hours and 24 hours after the acute challenge.

Moreover, at molecular level, we focus on neuronal activation, in terms of *Arc* and *Gadd45β* expression, and on neuroplastic mechanisms, by measuring the neurotrophin *Bdnf*, since it is well known the strong relationship between acute stress response and neuroplasticity (Calabrese et al., 2009). The analyses were conducted after the exposure to the acute stress but also after the test, in order to evaluate if the behavioral phenotype is associated with alterations due to the challenge per sè, or/and to modification of the system set in motion during the cognitive performance.

The molecular study was performed in prefrontal cortex and dorsal hippocampus, two brain regions known to be connected with working memory and with the response systems implicated in the coping ability with external and internal challenges.

1.18.1 Results

1.18.1.1 Effect of one hour of acute restraint stress on the cognitive performance in the novel object recognition (NOR) test

As shown in figure 33, one-way ANOVA revealed a significant stress effect ($F_{3\text{-}24}$:3.790, $p<0.05$). Animals exposed to the novel object recognition test performed significantly better when examined at 1hour (+100%, $p<0.05$ vs no stress-NOR) and at 4 hours (+76%, $p<0.05$ vs no stress-NOR) post- stress compared to control animals. This difference disappeared 24 hours after the acute stress, when rats performed like the non-stressed rats (+1%, $p>0.05$ vs no stress-NOR).

These results indicate that acute stress facilitates working memory within the time frame of 1 to 4 hours.

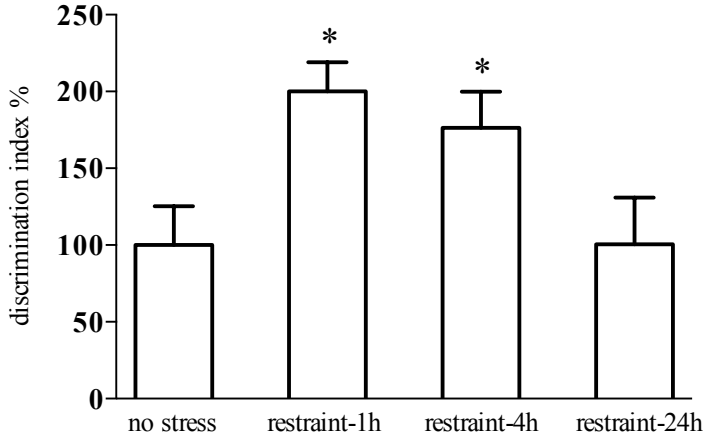

Fig. 33: Effect of one hour of acute restraint stress on the cognitive performance, measured in the novel object recognition test, 1h (restraint-1h), 4h (restraint-4h) and 24h (restraint-24h) after the end of the challenge. The data, expressed as discrimination index % of no stress rats (set al 100%), are the mean of at least 7 independent determinations ± SEM. * $p<0.05$, vs no stress (one-way ANOVA with PLSD).

1.18.1.2 Effect of one hour of acute restraint stress on Arc expression in rat prefrontal cortex and dorsal hippocampus

Focusing on the immediate early gene Arc, we evaluated the effect of neuronal activation, in response to one hour of acute restraint stress to establish its possible contribution to the behavioral results observed.

In prefrontal cortex (fig. 34A) we found a significant effect of stress ($F_{3\text{-}22}$:9.952, $p<0.001$), with Arc mRNA levels being up-regulated 1 hour (+173%, $p<0.001$ vs no stress), 4 hours (+ 74%, $p<0.05$ vs no stress) and 24 hours (+87%, $p<0.05$ vs no stress) after the end of the acute challenge, in comparison to non-stressed group.

Similarly, in the dorsal hippocampus (fig. 34B), one-way ANOVA reveled a significant effect of the stress ($F_{3\text{-}22}$:3.813, $p<0.05$). Accordingly, Arc gene expression increased in restraint-1h group (+38%, $p<0.05$ vs no stress), and restraint 24h (+40%, $p<0.05$ vs no stress) with respect to control animals, whereas the slight increase in restraint-4h did not reach the statistical significance (+20, $p>0.05$ vs no stress).

These results, in both the brain regions, indicated that acute stress caused a rapid induction of the IEGs expression that last until 24 hours later the one hour of restraint.

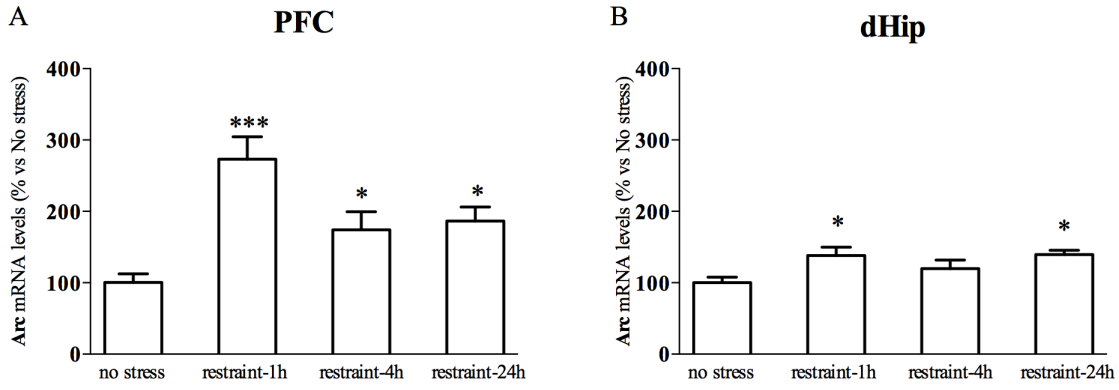

Fig. 34: Analysis of Arc mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of acutely stressed rats sacrificed 1h (restraint-1h), 4h (restraint-4h) and 24h (restraint-24h) after the end of the challenge. The data, expressed as % of no stress rats (set al 100%), are the mean of at least 5 independent determinations ± SEM. * $p<0.05$, *** $p<0.001$ vs no stress (one-way ANOVA with PLSD).

1.18.1.3 Effect of one hour of acute restraint stress on Gadd45β expression in rat prefrontal cortex and dorsal hippocampus

Next, we assessed the modulation of the acute challenge on the activity-dependent immediate early gene *Gadd45β.* In both the brain regions, we found a significant effect of stress (PFC: F_{3-22}: 22.178, $p<0.001$; dHip F_{3-22}: 13.545, $p<0.001$, one-way ANOVA),with an up-regulation of *Gadd45β* specifically one hour after the stress (PFC: +115%, $p<0.001$ vs no stress; dHip: +63%, $p<0.001$) (fig. 35A-B), whereas only in dHip the modulation is still present 24h after the stress (+25%, $p<0.05$ vs no stress) (fig. 35B).

The results obtained, indicated that the effect of the restrain protocol on *Gadd45β* is transient, probably partially contributing to the behavioral outcome.

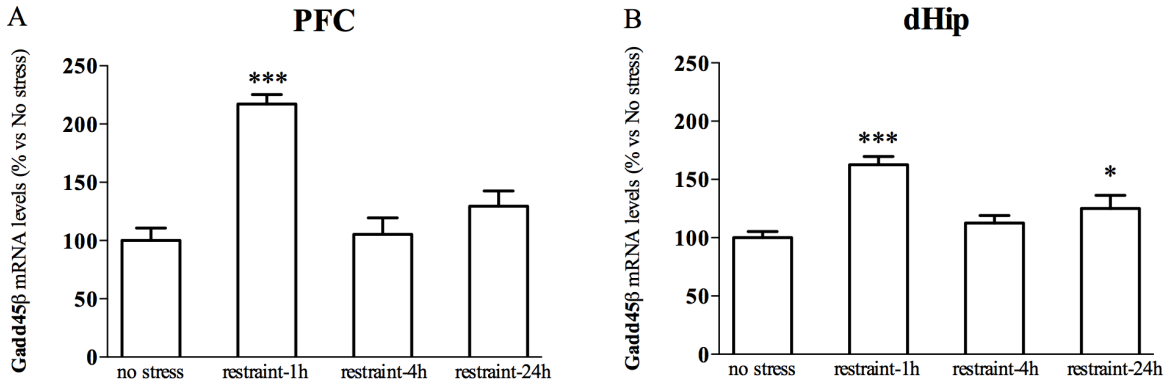

Fig. 35: *Analysis of Gadd45β mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of acutely stressed rats sacrificed 1h (restraint-1h), 4h (restraint-4h) and 24h (restraint-24h) after the end of the challenge.* The data, expressed as % of no stress rats (set al 100%), are the mean of at least 5 independent determinations ± SEM. * $p<0.05$, *** $p<0.001$ vs no stress (one-way ANOVA with PLSD).

1.18.1.4 Effect of one hour of acute restraint stress on total Bdnf and Bdnf isoform IV expression in rat prefrontal cortex and dorsal hippocampus

In the prefrontal cortex, the one-way ANOVA analysis showed a significant effect of stress (F_{3-22}: 8.523, $p<0.01$) on total *Bdnf* expression (fig. 36A). Indeed, total Bdnf mRNA levels significantly increased 1h after the stress (+66%, $p<0.001$ vs no stress).

Similarly, acute restraint stress significantly modulated *Bdnf* isoform IV (F_{3-22}: 20.343, $p<0.001$). Accordingly, one hour of stress induced an up-regulation at 1h (+149%, $p<0.001$ vs no stress) and 24h (+46%, $p<0.05$ vs no stress) (fig. 36B).

In dorsal hippocampus, we did not find a statistic effect of stress in the one-way ANOVA analysis (F_{3-22}: 2.242, $p>0.05$) for total *Bdnf.* Nevertheless, as shown in figure 36C, 24 hours after the acute challenge total *Bdnf* were significantly increased in comparison with non-stressed rats (+59%, $p<0,05$ vs no stress).

In this brain region, the levels of the *Bdnf* isoform IV (fig. 36D) were not significantly affected by the stress (F_{3-22}: 0.171, $p>0.05$).

These results indicate that the improvement in the cognitive performance observed one hour after the acute stress required the up-regulation of *Bdnf* specifically in the PFC.

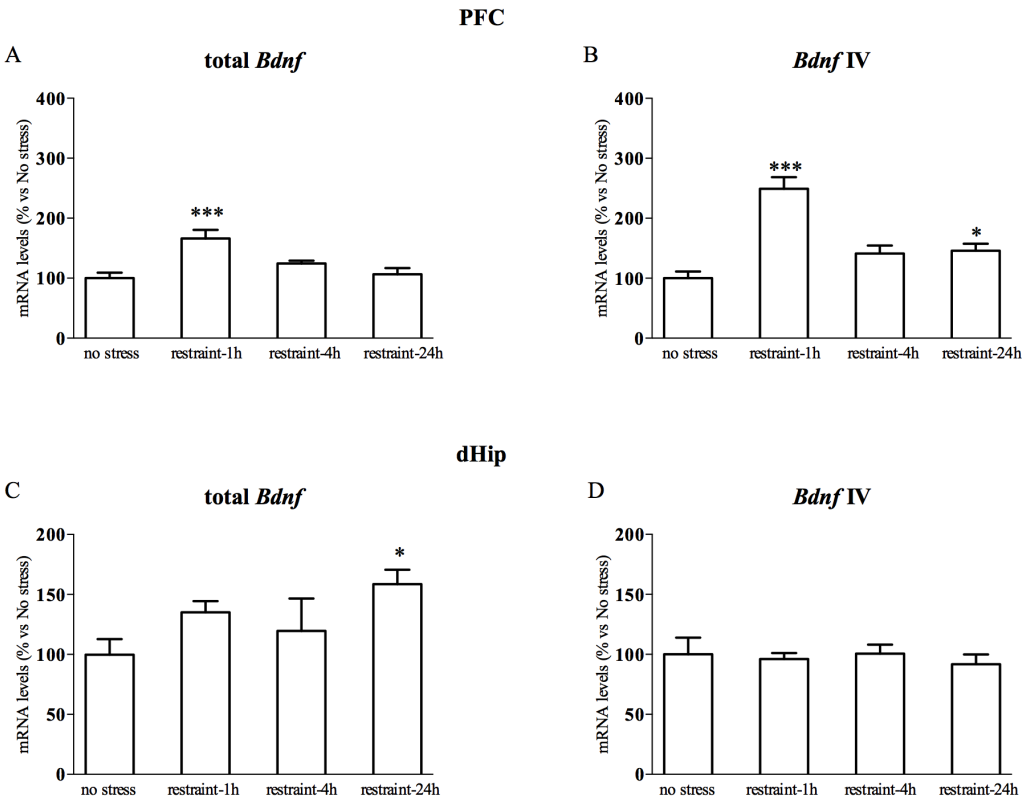

Fig. 36: Analysis of total Bdnf and Bdnf isoform IV mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of acutely stressed rats sacrificed 1h (restraint-1h), 4h (restraint-4h) and 24h (restraint-24h) after the end of the challenge. The data, expressed as % of no stress rats (set al 100%), are the mean of at least 5 independent determinations ± SEM. * $p<0.05$, *** $p<0.001$ vs no stress (one-way ANOVA with PLSD).

1.18.1.5 Effect of the cognitive test exposure after the acute restraint stress: focus on Arc expression in rat prefrontal cortex and dorsal hippocampus

In prefrontal cortex (fig. 37A), we found a significant effect of the stress ($F_{3\text{-}50}$: 4.678, $p<0.01$), of the cognitive test ($F_{1\text{-}50}$: 104.936, $p<0.001$) and of the stressXtest interaction ($F3_{\text{-}50}$: 6.375, $p<0.01$). Indeed, the exposure to NOR test induced a significant increase of *Arc* not only in non-stressed rats (+264%, $p<0.001$ vs no stress/Naïve) but also in the groups tested 4 hours (+96%, $p<0.001$ vs restraint-4h/Naïve) and 24 hours (+173%, $p<0.001$ vs restraint-24h/Naïve) after the acute challenge. Actually, one hour after the acute challenge, the exposure to the cognitive test did not lead to an additional increase of *Arc* mRNA levels (+25%, $p>0.05$ vs restraint-1h/Naïve), thus implicating that at this time point the system is enough functioning or too activated to induce a further up-regulation during the cognitive task.

On the contrary, in dorsal hippocampus two-way ANOVA analysis revealed a significant effect only of the cognitive test ($F_{1\text{-}49}$: 13.720, $p<0.01$), but not of the stress ($F_{3\text{-}49}$: 1.249, $p>0.05$) and of the stressXtest interaction ($F_{3\text{-}49}$: 1.496, $p>0.05$), with *Arc* being up-regulating only in no stress rats (+64%, $p<0.01$ vs no stress/Naïve) (fig. 37B).

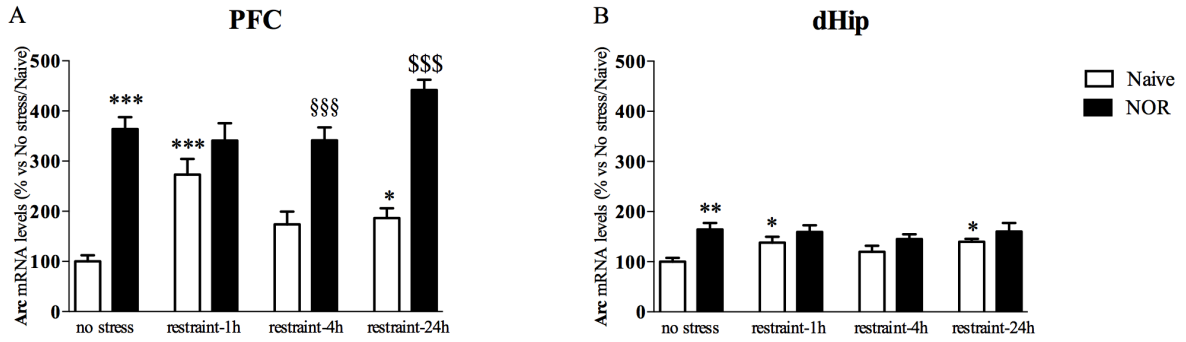

Fig. 37: Analysis of Arc mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of acutely stressed rats exposed to the novel object recognition test (NOR) 1h (restraint-1h), 4h (restraint-4h) and 24h (restraint-24h) after the end of the challenge. The data, expressed as % of no stress/Naive rats (set al 100%), are the mean of at least 5 independent determinations ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs no stress/Naive; §§§ $p<0.001$ vs restraint-4h/Naive; $$$ $p<0.001$ vs restraint-24h/Naïve (two-way ANOVA with PLSD).

1.18.1.6 Effect of the cognitive test exposure after the acute restraint stress: focus on Gadd45β expression in rat prefrontal cortex and dorsal hippocampus

In prefrontal cortex, two-way ANOVA results showed a significant effect of stress ($F_{3\text{-}50}$: 8.089, $p<0.001$), of the cognitive test ($F_{1\text{-}50}$: 92.221, $p<0.001$) and of stressXtest interaction ($F_{3\text{-}50}$: 8.884, $p<0.001$). Accordingly, the exposure to the behavioral test induced an up-regulation of Gadd45β mRNA levels in non-stressed rats (+116%, $p<0.001$ vs no stress/Naïve), in rats tested 4hours (+133%, $p<0.001$ vs restraint-4h/Naïve) and 24 hours (+77%, $p<0.001$ vs restraint-24h/Naïve) after the stress.

However, one hour after the acute challenge we did not observed any further up-regulation induced by the NOR (: +5%, $p>0.05$ vs restraint-1h/Naïve) (fig. 38A).

On the contrary, in dorsal hippocampus (fig. 38B), we found a different modulation of Gadd45β following the NOR task. Indeed, in line with previous findings (chapter 4.2, fig. 19B), the cognitive performance induced a significant increase of its mRNA levels (+63%, $p<0.001$ vs no stress) in non-stressed rats, while the exposure to the test one hour after the acute challenge significant decreased Gadd45β expression (-28%, $p<0.05$ vs restraint-1h/Naïve), as confirmed by the two-way ANOVA analysis (test: $F_{1\text{-}50}$: 4.169, $p<0.05$; stressXtest: $F_{3\text{-}50}$: 11.122, $p<0.05$).

These results further support that the prefrontal cortex is the brain region mainly activated during the cognitive performance. Again, we did not find this increased activation compare to the "basal" condition in the restrain-1h group, as confirmation of the fact that the improvement in the cognitive performance found at this time point is due to the effect of the acute stress.

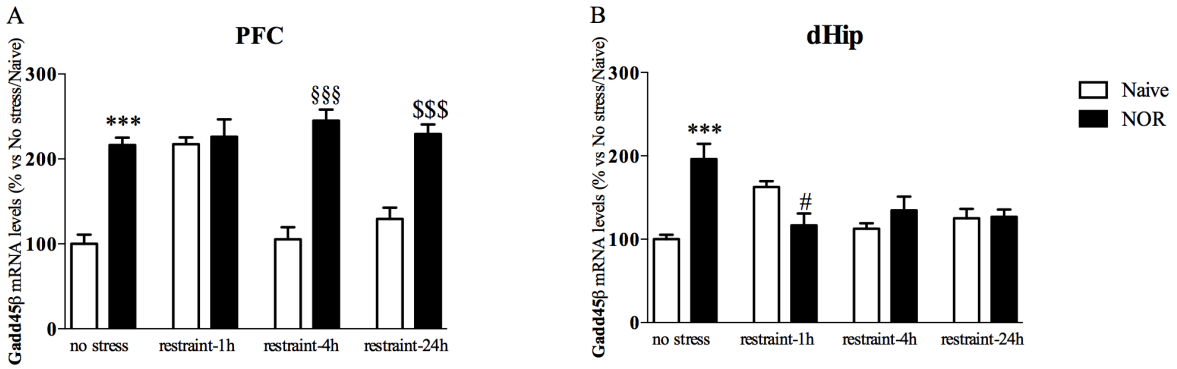

Fig. 38: Analysis of Gadd45β *mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of acutely stressed rats exposed to the novel object recognition test (NOR) 1h (restraint-1h), 4h (restraint-4h) and 24h (restraint-24h) after the end of the challenge.* The data, expressed as % of no stress/Naive rats (set al 100%), are the mean of at least 5 independent determinations ± SEM. * $p<0.05$, *** $p<0.001$ vs no stress/Naive; # $p<0.05$ vs restraint-1h, §§§ $p<0.001$ vs restraint-4h/Naïve; $$$ $p<0.001$ vs restraint-24h/Naïve (two-way ANOVA with PLSD).

1.18.1.7 Effect of the cognitive test exposure after the acute restraint stress: focus on total Bdnf and Bdnf isoform IV expression in rat prefrontal cortex and dorsal hippocampus

In prefrontal cortex, two-way ANOVA results showed a significant effect of stress ($F_{3\text{-}48}$: 4.147, $p<0.05$) and of the cognitive test ($F_{1\text{-}48}$: 29.815, $p<0.001$), with no significant stressXtest interaction ($F_{3\text{-}48}$: 0.672, $p>0.05$) on total *Bdnf* expression. Accordingly, the behavioral test induced an up-regulation of total *Bdnf* mRNA levels in non-stressed rats (+83%, $p<0.001$ vs no stress/Naïve), in rats tested 4hours (+67%, $p<0.01$ vs restraint-4h/Naïve) and 24 hours (+52%, $p<0.05$ vs restraint-24h/Naïve) after the challenge (fig. 39A).

Similar to what observed for the total form of the neurotrophin, *Bdnf* isoform IV was significantly modulated by stress ($F_{3\text{-}50}$: 12.874, $p<0.001$) by the cognitive test ($F_{1\text{-}50}$: 40.345, $p<0.001$) and by the stressXtest interaction ($F3_{\text{-}50}$: 3.525, $p<0.05$). Indeed, as shown in figure 39B, the test exposure significantly increased *Bdnf* isoform IV in no stress (+144%, $p<0.001$ vs no stress/Naïve), in restraint-4h (+45%, $p<0.05$ vs restraint-4h/Naïve) and in restraint-24h (+56%, $p<0.01$ vs restraint-24h/Naïve) groups.

On the contrary, both for total *Bdnf* and for *Bdnf* isoform IV, one hour after the acute challenge we did not observed any further up-regulation induced by the cognitive test (total *Bdnf*: +27%, $p>0.05$ vs restraint-1h/Naïve; *Bdnf* isoform IV: +14%, $p>0.05$ vs restraint-1h/Naïve).

In dorsal hippocampus we found a milder modulation due to both stress and cognitive test.

For total *Bdnf* (fig. 39C) we observed a significant stressXtest interaction ($F_{3\text{-}49}$: 3.093, $p<0.05$), with no effect of the stress ($F_{3\text{-}49}$: 1.023, $p>0.05$) and of the test ($F_{1\text{-}49}$: 2.579, $p>0.05$). The post hoc analysis, indeed, showed a significant increase of total *Bdnf* only in non-stress rats exposed to NOR (+50%, $p<0.05$ vs no stress/Naïve).

On the contrary, *Bdnf* isoform IV was not modulated by the stress ($F_{3\text{-}50}$: 0.659, $p>0.05$), by the test ($F_{1\text{-}50}$: 0.346, $p>0.05$), with no significant stressXtest interaction ($F_{3\text{-}50}$: 0.444, $p>0.05$) (fig. 39D).

These results suggest that the neurotrophin increased in the PFC may be implicated in the correct cognitive performance. Instead, as observed for *Arc*, the up-regulation induced by the acute stress per sè that is already present 1 hour later is sufficient to significantly improve the results in the behavioral test.

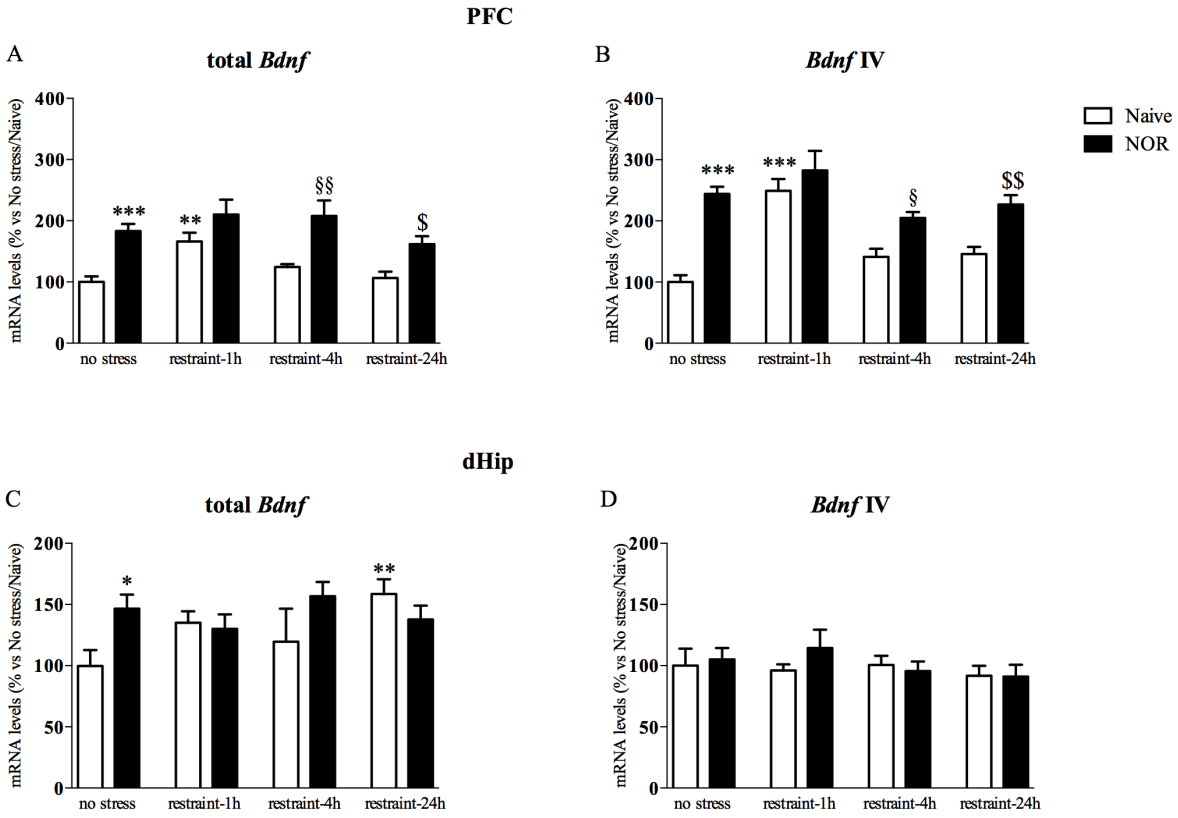

Fig. 39: Analysis of total Bdnf and Bdnf isoform IV mRNA levels in the prefrontal cortex (PFC) and dorsal hippocampus (dHip) of acutely stressed rats exposed to the novel object recognition test (NOR) 1h (restraint-1h), 4h (restraint-4h) and 24h (restraint-24h) after the end of the challenge. The data, expressed as % of no stress/Naive rats (set al 100%), are the mean of at least 5 independent determinations ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs no stress/Naive; § $p<0.05$, §§ $p<0.01$ vs restraint-4h/Naive; $ $p<0.05$, $$ $p<0.01$ vs restraint-24h/Naïve (two-way ANOVA with PLSD).

1.18.2 Discussion

In this study we found that exposure to acute stress positively acts on cognitive performance with a specific time profile. Moreover, at molecular level our results support that neuroplastic mechanisms play a fundamental role in working memory processes as well as in the improvement due to a single session of restraint stress.

The role of stress in modulating learning and memory has been well described (Joels et al., 2006), with different issues based on the type and length of stressors. Indeed, while chronic stress has detrimental effect on cognitive functions (McEwen and Sapolsky, 1995), acute stress may have a dual effect: it may improve memory or, when severe, it can impair it (Hains and Arnsten, 2008). In the field of adaptive response to stress, several studies have shown that stress, in close association with learning task, facilitated the memory consolidation (de Kloet et al., 1999) and might be indispensable for the good learning (Sandi et al., 1997;Lupien et al., 2002). Here, we assessed the influence of one hour of acute restraint stress in modulating the cognitive performance measured 1 hour, 4 hours and 24 hours post challenge. We found that animals exposed to the restraint stress performed significantly better than non-stressed rats when examined 1 hour and four hours post-stress. This difference disappeared 24 hours following the challenge, indicating a transient effect of stress pre-exposure and the performance. In accordance, Yuen and colleagues demonstrated an enhanced working memory in the T-maze following 20 minutes of forced swim stress not only 4 hours but also 1 day after, whereas not 48 hours later (Yuen et al., 2009). The discrepancy between the effect found 24 hours after the challenge may be due to the different stress used and to the diverse cognitive task employed. Additionally, others behavioral studies demonstrated that moderate acute stress facilitated classical fear conditioning (Shors et al., 1992).

Then, in order to establish the molecular mechanisms set in motion by the acute stress, potentially involved in promoting the performance in the novel object recognition test, here we focused on neuronal activation and neuroplasticity.

The immediate early gene *Arc* was highly enhanced by the restraint stress, in both prefrontal cortex and dorsal hippocampus, and this modulation last until 24 hours after the stress, suggesting a long-lasting activation driven by the challenge. However, the higher increase at time 1 hour, as the results obtained for the neuronal activity dependent gene Gadd45β at the same time point, in both the brain regions, suggested the possible implication of the IEGs in the behavioral results. Accordingly, in the PFC, the increase of total *Bdnf* and *Bdnf* isoform IV occurred rapidly after 1 hours of stress and it was transient, in line with the evidence the neurotrophin is enhanced by acute stimulations, thus being a part of a compensatory response to preserve brain homeostasis (Marmigere et al., 2003). Indeed, a short stress may trigger the

neurotrophin increase thus contributing to store information that could serve to prepare a response to a new stimulus. Furthermore, the fact that 4 and 24 hours post-stress the neurotrophins were not still increased may suggest that in the PFC the acute challenge enhance mainly short-time effect in the *Bdnf*-early response. Conversely in the dHip, total *Bdnf* was significantly modulated 1day post-stress, indicating a different brain region involvement in the fast stress response. On the contrary, Marmigere and colleagues found that short-time stress application (60 minutes) increased *Bdnf* in the whole hippocampus, whereas a longer time of stress (180 minutes) decreased it (Marmigere et al., 2003). Moreover, when we assessed the molecular outcome in the animals exposed to the NOR, we found that in the PFC the IEGs *Arc* and *Gadd45β* were significantly up-regulated in non-stressed rats and in animals tested 4 and 24 hours post stress, in accordance with the evidence that the immediate early genes play a role in the neuroplastic mechanisms required for the memory consolidation processes (Robertson, 1992;Dragunow, 1996;Tischmeyer and Grimm, 1999). Indeed, the inhibition of the hippocampal Arc protein expression impaired long-term potentiation as well as the long term memory (Guzowski et al., 1999), supporting the findings that the *Arc*-mediated improved plasticity we observed could be involved in the behavioral outcome obtained in this experiment. Similarly, the expression of both the total neurotrophin and *Bdnf* isoform IV was significantly enhanced in the cognitive task in non-stressed rats and in stressed rats tested in the NOR 4 and 24 hours following the acute stress. Accordingly, it is known that neurotrophic factors are implicated in long term potentiation and that stress may modify cognitive function via regulation of Bdnf (Dragunow et al., 1993).

The present findings suggest that acute restraint stress per sè was able to up-regulate the gene expression of different target up to 24 hours later in both the brain regions with a different time-profile. Moreover, these increases were also observed after the NOR.

Interestingly, one-hour after the restraint stress, when the improvement in the cognitive performance was major, we did not find a further regulation due to the test (compare to that caused by the stress) suggesting that starting the test with an already high level of these genes is enough to ameliorate the behavioral outcome.

Summary and conclusions

In conclusion, the results obtained during my PhD provide evidence, at preclinical level, about the effects of stress exposure at adulthood in contributing to the development of mood disorders. We used different experimental paradigms not only to investigate the molecular mechanisms contributing to the development of the depressive like-behavior, but also to identify pathways and systems related to remission and to relapse, which are critical aspects for the treatment of depressed patients. Moreover, we add knowledge on the molecular effect of the antipsychotic drug lurasidone, characterized by a multi-receptors profile, in the modulation of the stress-induced alterations.

We took advantage of the chronic mild stress paradigm, a well-established animal model of depression, to analyze the vulnerability to stress exposure and we demonstrated that independently from the susceptible and resilient phenotype, in term of anhedonia, all stressed rats developed cognitive deficits. These behavioral results suggested that the molecular systems underlying several pathological domains of MDD are probably different. In particular, we found that the cognitive dysfunctions were associated with the inability to activate the synthesis of new proteins specifically at synaptic levels in rat dorsal hippocampus, a key brain region for memory processes. More in details, we have shown that chronic stress interfered with the elongation phase of the protein synthesis, by preventing the activation of the NMDA-mTOR pathway, a fundamental step for the correct cognitive performance that we observed in non-stressed animals (Calabrese et al., 2017).

Furthermore, we provide further evidence of the involvement of the hypothalamic-pituitary-adrenal axis, and in particular of the genomic and non-genomic effects of glucocorticoid receptor in the dorsal hippocampus, in the CMS-induced behavioral abnormalities. Indeed, we demonstrated that the activation of the GR nuclear signaling was associated with the correct cognitive performance whereas chronic stress exposure interfered with this mechanism, thus inhibiting the transcription of GR responsive genes involved in learning and memory processes. In addition, stress, by increasing the availability of GR at membrane levels, seems to direct preferentially the action of hormones more towards the non-genomic pathways, thus altering synaptic and mitochondrial signaling.

Moreover, in a section of this thesis, we considered the implication of epigenetic mechanisms in the regulation of the HPA axis in the CMS-mediated behavioral deficits and we found that chronic stress exposure increased the DNA methylation of specific genes involved in the HPA axis signaling, thus interfering with their transcription, in rat prefrontal cortex. As an example, we found that 7 weeks of CMS increased the methylation status of the CGs in the glucocorticoid

responsive element on Gadd45β DNA sequence, underlying the inability of the glucocorticoid receptor to activate its downstream target.

Furthermore, we showed that the effects of chronic stress exposure during adult life were persistent, with long-lasting consequences not only on epigenetics, with the GR responsive factors Gadd45β and Sgk1 being hypermethylated also after the recovery, but also on mechanisms of neuronal plasticity, including the neurotrophic factor Bdnf, suggesting that not all the systems impaired by stress were restored during remission, thus representing some scars of vulnerability that, in turn, may promote the relapse to the pathology.

Moreover, since a high percentage of depressed patients experience relapse, condition that may be due to the inability to correctly react to any external stimuli even when the recovery is achieved, we investigated the outcomes of a novel stressor exposure after the remission phase. In particular, we observed that the long-lasting effect of stress on *Bdnf* expression in prefrontal cortex interfered with the capability to react to a new challenge in the post stress period, suggesting the endurable detrimental consequence of chronic adversities. On the contrary, we found that the increased expression of activity-dependent genes, such as Arc, induced by an acute stress was not impaired by a previous exposure to CMS, indicating that different mechanisms control the persistent modifications induced by chronic stress.

Conversely, the exposure to one hour of acute restraint stress improved cognitive function with a specific temporal profile. Indeed, we found that the challenge enhanced the performance one hour and four hours post the acute stressor. Interestingly we pointed out how this adaptive response was mediated by the induction of mechanisms of neuronal plasticity mainly in the rat prefrontal cortex. Indeed, we demonstrated that the increased expression of the activity dependent early genes and of Bdnf, due to the acute stress, were responsible for the behavioral outcome in the novel object recognition task, thus underlying their fundamental contribution in the memory performance.

We underlined the ability of lurasidone in modulating the behavioral alterations induced by the CMS paradigm. Indeed, the drug was able to normalize the anhedonic phenotype, in line with our previous findings (Calabrese et al., 2016), and completely reverted the cognitive deficits due to chronic stress exposure. This result provides further support to the pro-cognitive effect of lurasidone, that may be mediated by its intrinsic activity as antagonist of the serotoninergic receptor 5HT-7, known to be important for learning and memory (Hedlund and Sutcliffe, 2004;Thomas and Hagan, 2004).

At molecular level, we highlighted the ability of this multi-receptor modulator drug in normalizing the anhedonic phenotype and the cognitive deficits by restoring the modification observed in the GR activity. In particular, lurasidone not only acted at genomic levels, but

exerted protective properties on the membrane GR pathways at both synaptic and mitochondrial levels, thus counteracting the stress-related abnormalities. Moreover, chronic lurasidone treatment was able to normalize DNA methylation changes produced by chronic stress, suggesting the potential of the drug to interfere with the epigenetic alterations produced by the adverse experiences.

Conversely, if lurasidone treatment is interrupted during the rest period, the protective effect is lost, indicating that the continuation of the pharmacological administration even after the end of the stress phase, may avoid the possible rebound mechanisms set in motion by drug withdrawal.

Indeed, it may be proposed that the extension of the treatment during the recovery could have a beneficial effect in preventing further relapses. However, when the stressed brain had to react to a new challenge following the stress and treatment washout, the administration of lurasidone in the pre-recovery period was able to counteract some alterations due to chronic stress, by facilitating the reaction to the new stimulus. In particular, with respect to the neurotrophic factor, the drug had the ability to enhance the transcription of the neurotrophin in rats previously stressed, indicating the protective efficacy of lurasidone in counteracting CMS-induced scars.

In conclusion, the results obtained during my PhD program contribute to better understand the maladaptive and adaptive outcomes of stress exposure during adult life. We describe potential molecular mechanisms that contribute to different aspects and phases of stress-related disorders. In particular, we highlight the complexity of the changes contributing to the long-lasting functional effects brought about by stress exposure that may, therefore, be relevant for specific domains of psychiatric disorders. In addition, even if the involvement of the glucocorticoid receptor in the development of psychiatric disorders is well established, we demonstrated, for the first time, the ability of lurasidone to counteract the detrimental consequence of chronic stress exposure, by modulating the HPA axis functioning, mechanisms by which the antipsychotic lurasidone may act as antidepressant.

However, the use of one drug with a multi-receptor profile in a specific animal model of the pathology might be considered as a limitation of the study. It will be important to investigate drugs with different receptor profiles in order to establish the different effects on distinct functional domains affected by stress exposure.

Furthermore, the employment of selected behavioral tests with a precise schedule allowed us to evaluate specific symptomatologic domains of stress-related disorders, although additional tests will be necessary to provide a more complete picture of the changes that may contribute to the risk of psychiatric disorders.

All in all, our findings add new knowledge to the field of the pharmacological research for novel targets and approaches for the treatment of depression and stress-related disorders.

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