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THE RAPID AND LONG-TERM IMPACT OF ACUTE STRESS ON NEUROPLASTICITY. A NOVEL PARADIGM FOR DISSECTION OF THE STRESS RESPONSE

BIO/14

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English abstract

The response to stressful events is physiologically required to adapt to external challenges. However, the stress response can either have a 'pro-adaptive' outcome, when the response is efficiently activated and then inactivated, or exert 'maladaptive' effects, when the subject is vulnerable and the response is dysregulated. Unfortunately, it is still unknown what mechanisms address the individual responses towards pro-adaptive or maladaptive outcome of stress.

In previous studies we found that acute inescapable footshock (FS)-stress rapidly enhances depolarization-evoked glutamate release from prefrontal and frontal cortex (PFC/FC) purified synaptic terminals (synaptosomes), with a mechanism involving rapid, non-genomic enlargement of the readily releasable pool (RRP) of glutamate presynaptic vesicles. Moreover, we showed that the same protocol of acute stress induces long-term atrophy/remodeling of apical dendrites in the same area up to 14 days later, thus suggesting that the consequences of acute stress are far from being simply acute.

Traditional models of chronic stress reproduce only the endpoint of several changes occurring in brain and body during the stress response. On the opposite, analyzing short- and long-term alterations induced by acute stress could be a useful tool to dissect adaptive and maladaptive components underlying stress response.

Here, we aimed at dissecting the early and delayed alterations induced by a single session of acute FS-stress, focusing on the putative effectors that trigger towards adaptive/maladaptive trajectories of the stress response.

We demonstrated that FS-stress increases both glutamate release evoked by depolarization and RRP size up to 24h after the stress protocol. In parallel, we found that acute FS-stress exerts different time-dependent molecular alterations in synaptic membranes from PFC/FC. We then set up a behavioral paradigm that discriminates resilience/vulnerability towards acute stress, based on stress-induced behavioral alterations.

We demonstrated that the different behavioral phenotypes are associated with specific functional and molecular alterations in PFC glutamatergic synapses.

Our approach, dissecting rapid and delayed effects of acute stress response, and their adaptive/maladaptive components, could lead to better understand mechanisms that turn stress response from a physiological adaptation into maladaptive alterations. The final aim is to look for the putative effectors of this switch, searching for markers of vulnerability and novel targets for the therapies of neuropsychiatric disorders.

Italian abstract

La risposta ad eventi stressanti è un processo fisiologico necessario per l'adattamento all'ambiente e per la sopravvivenza. Tuttavia, la risposta allo stress può essere adattativa, quando è attivata e poi disattivata in modi e tempi adeguati, oppure maladattativa, quando il soggetto che la mette in atto è vulnerabile e/o la risposta è inadeguata.

In studi precedenti, abbiamo dimostrato che un singolo episodio stressante (footshock inevitabile) incrementa il rilascio di glutammato da terminali sinaptici siti nella corteccia prefrontale/frontale (PFC/FC) con un meccanismo che implica l'aumento, rapido e regolato da meccanismi non genomici, del pool di vescicole di glutammato a pronto rilascio (readily releasable pool, RRP). Inoltre, abbiamo dimostrato che lo stresso protocollo di stress acuto induce atrofia e rimodellamento dendritico sempre nella corteccia prefrontale. Tali alterazioni morfologiche sono mantenute almeno fino a 14 giorni dopo il protocollo di stress acuto, suggerendo che gli effetti dello stress acuto potrebbero essere molto più che semplicemente rapidi e transienti.

I modelli tradizionali impiegati per lo studio di patologie neuropsichiatriche sono basati sull'utilizzo di un paradigma di stress cornico, ma in realtà riproducono solo l'effetto finale delle diverse alterazioni messe in atto dal cervello e dall'organismo per rispondere allo stress. Al contrario, osservare gli effetti a breve e lungo termine dello stress acuto potrebbe essere un utile mezzo per distinguere le componenti adattative e maladattative sottese alla risposta allo stress.

In questo progetto, il nostro scopo è stato quello di studiare se le alterazioni funzionali e molecolari rapide indotte dal footshock siano anche sostenute nel tempo (nelle prime 24h dopo il paradigma stressante) e di provare ad individuare quali siano i possibili fattori che indirizzano la risposta allo stress verso un processo adattativo o uno maladattivo.

Prima di tutto, abbiamo dimostrato che lo stress acuto induce un incremento della dimensione del RRP e un aumento di rilascio evocato di glutammato che sono rapidi e mantenuti fino a 24h dopo il footshock. Inoltre, abbiamo mostrato che i cambiamenti funzionali sono accompagnati da diverse alterazioni molecolari in membrane sinaptiche nell'arco delle 24h.

Successivamente abbiamo ideato un paradigma che permettesse di distinguere animali vulnerabili o resilienti allo stress acuto, sulla base della comparsa di alterazioni comportamentali 24h dopo il footshock.

Abbiamo dimostrato che i soggetti stressati mostrano alterazioni funzionali e molecolari delle sinapsi glutammatergiche che sono specifiche del fenotipo comportamentale individuato.

Il nostro approccio, che studia le alterazioni nel tempo dello stress acuto e prova a distinguerne le componenti adattative e maladattative, potrebbe permettere di comprendere quali siano i meccanismi che trasformano la fisiologica risposta adattativa allo stress in un processo maladattativo. Lo scopo finale è quello individuare i marker di vulnerabilità allo stress e nuovi target farmacologici per la terapia di malattie neuropsichiatriche.

Summary

1 Introduction

1.1 The stress response

1.1.1 The concepts of homeostasis and allostasis

All organisms happen to face predictable alterations (e.g. light and darkness daily cycles, seasonal changes) and unpredictable events, including potential stressors, that require immediate physiological and behavioral adjustments. Evolutionary, the stress response is the physiological mechanism that allows adaptation to real or perceived environmental and homeostatic challenges. An adequate stress response, involving the perfect tuning of neuroendocrine and autonomic systems, is required for survival [McEwen and Wingfield, 2010; Ulrich-Lai and Herman, 2009].

The idea of *homeostasis* has been historically introduced to describe the adjustments required for survival in a changing environment. However, the concepts of stress and homeostasis have been used ambiguously, without considering all the processes involved and lacking the proper attention to several aspects affecting the ability to cope, such as the experiences and the genetic background of the subject. To fill this gap, McEwen and Wingfield proposed the new concept of *allostasis*, defined as the ability to achieve stability through change. Allostasis is the fundamental process through which organisms actively adjust to both predictable and unpredictable events. This new idea helps distinguishing between the systems that are necessary for life (i.e. that maintain the steady state, concept of "homeostasis") and those that preserve these systems in balance (concept of "allostasis") [McEwen and Wingfield, 2003; McEwen and Wingfield, 2010]. Thus, homeostasis is the stability of a limited number of systems that need to be maintained within an optimal range for the current life stage, such as pH, body temperature, glucose levels, and oxygen tension. Instead, we can refer to allostasis considering the mobilization of glucocorticoids and catecholamines during physical activity, the rise of blood pressure when we stand up or the shift in metabolism before and during hibernation [McEwen and Wingfield, 2003]. Chronic

allostatic state or the inability to adequately turn on, and then turn off allostatic systems produce a load on the body. This can be considered as the cumulative outcome of all the alterations that an organism applies to cope and is termed *allostatic load*. To a certain extent, these changes are adaptive responses. However, when the response is prolonged or hyperactivated, allostatic load increases dramatically (*allostatic overload*) increasing the risk to develop pathologies [McEwen and Gianaros, 2011] (see Figure 1.1, below).

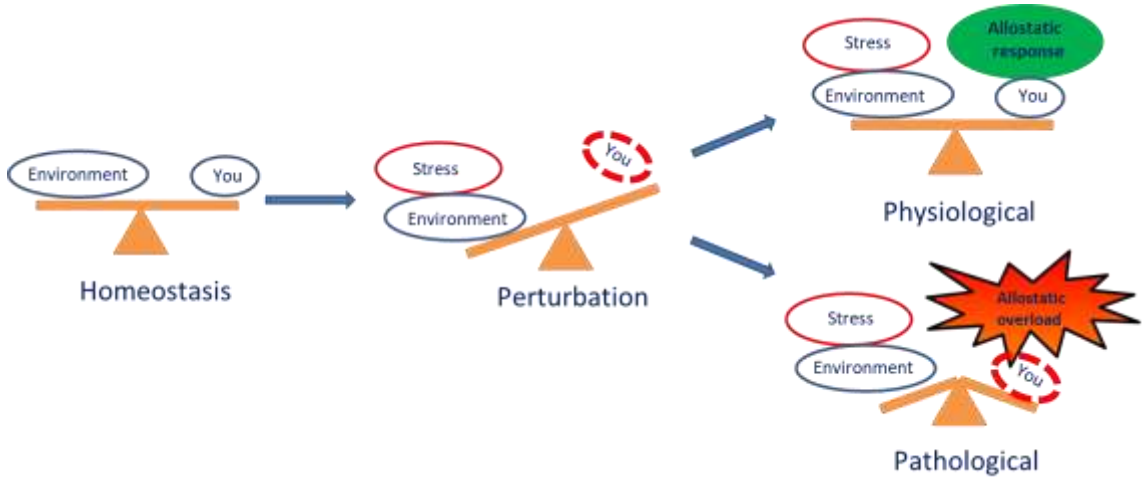

Figure 1.1: The concepts of stress, allostasis and allostatic overload. Stress is the perturbation of homeostasis and the stress response can either be adaptive and reestablish a new equilibrium state (allostatic response) or maladaptive, thus increasing the risk of developing pathologies (allostatic overload) [Adapted from McEwen, 2000].

1.1.2 Definition of stress

Nowadays the term stress is overused and ambiguous. In common language, stress is frequently used in the negative sense of "distress," referring to threatening or even traumatic events. Otherwise, it refers to the negative feeling typical of an intense and frantic life style. Even in the scientific community a clear and precise definition is lacking, thus presenting a main issue for researchers who study stress and the effects that it has on the brain and the body [Karastoreos, 2018]. The first scientist that tried to formulate a definition of stress is Hans Selye. The Canadian endocrinologist described stress as a nonspecific response of the body to any demand placed upon it [Selye, 1936]. In more recent literature, stress is defined as any condition that perturbs the physiological and psychological balance of an individual

[Franklin et al., 2012]. But, still, the common definition of "stress" focuses only on acute challenges with lack of attention to the complex network of the multiple systems involved and without including the responses activated when the subject is chronically exposed to stressors [McEwen, 2017]. Here we will use the definition of stress suggested by McEwen and Wingfield (2003): "the term stress will be used to describe events that are threatening to an individual and which elicit physiological and behavioral responses as part of allostasis in addition to that imposed by the normal life cycle".

1.1.3 The concepts of resilience and vulnerability and the role of the brain

After defining what stress is, it is worth answering to the following question: does everyone experience and deals with stress in the same way? Focusing on the effects that stress exert on a subject, we can classify stress as *good*, *tolerable* or *toxic* [McEwen and Wingfield, 2003].

We will call *good stress* or "eustress" any condition that, although representing a challenge or a risk for the subject, is well faced and has a positive and rewording outcome. "Tolerable stress" means, instead, that a negative event happens, but the subject has the personal resources and support systems to handle it. In these first two cases, the systems involved in the allostatic response are correctly turned on, and then cut off to promote adaptation. On the other hand, "toxic stress" refers to what happens when a subject lacks the capability of coping. In this last situation, the result is an increase in allostatic overload that can induce adverse effects on behavior and physiology [McEwen and Wingfield, 2003; McEwen 2018]. Hence, we can claim that "it's not stress that kill us, but how we deal with it" [Selye, 1936]. On one hand, some individuals, called here *resilient*, can perceive challenges as feasible or even poorly threatening and are able to enable adaptive responses. On the other hand, other subjects, that we will define *vulnerable*, fail in adapting to stressors and express inappropriate responses, that can increase the risk of developing pathologies [Franklin, 2012;

McEwen, 2014; Ebner and Singewald, 2017]. The biological basis underlying the individual differences in stress response and coping strategies are still poorly understood [Franklin, 2012]. However, it is largely accepted that the brain is the key organ in the stress response: it determines what the individual will perceive as stressful and possibly threatening, directs several systems of the body that are involved in short- and long-term responses (i.e. metabolic, cardiovascular, immune system) and orchestrates the behavioral health-promoting strategies (i.e. caloric intake, sleep, exercise) [McEwen, 2014; McEwen, 2017].

Moreover, the brain is a plastic organ that changes in its architecture, molecular profile and neurochemistry under both acute and chronic stress: the neural circuits in a healthy brain are remodeled by experiences to develop the appropriate responses for facing the situation [McEwen 2017]. Brain development and healthy neural function are required to establish an efficient response to challenges or stressors.

However, the vulnerable brain is less able to adapt properly, likely for these two reasons: it may not be so plastic, or it may have maladaptive circuitry or plasticity. The individual differences that allow these adaptive or maladaptive outcomes rely on the unique neurological capacity of the individual. This depend on genetic background but is also built upon experiences during the life course (i.e. environment, education, major life events), particularly those early in life [McEwen, 2014]. Figure 1.2, below, graphically represents the central role of brain in stress adaptive/maladaptive response [McEwen and Gianaros, 2011].

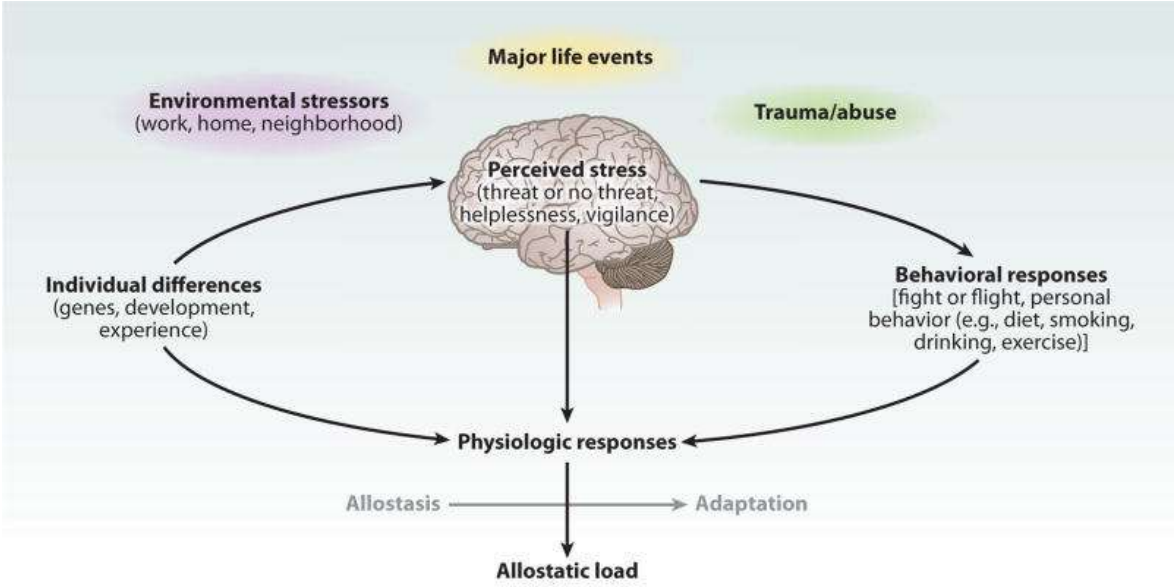

Figure 1.2: The central role of the brain in allostasis and the behavioral and physiological response to stressors [McEwen and Gianaros, 2011].

1.1.4 Gene-environment interaction in the stress response

Nowadays, identifying risk factors underlying individual vulnerability to stress-induced psychopathologies still represents a main challenge and a common goal. A role for genetic factors in stress vulnerability has been supported by meta-analysis and early biometrical genetic studies showing that stress-related psychopathologies aggregate in families and are moderately heritable [Kendler et al, 2006; Gatt et al., 2015]. Importantly, also more recent genome-wide studies of genetic variations confirm these findings [for a review see Smoller, 2016]. Some lines of clinical research claim that specific personality traits (i.e. "behavioral predispositions that reflect a reliable behavioral responsiveness of a given individual across time and circumstances") represent typical phenotypes of genetic variability [Weger, 2018]. As an example, high anxiety trait has been related to vulnerability to develop stress-induced psychopathology in humans and several preclinical studies try to adopt animal models genetically selected for their high or low anxiety to better assess this topic [Frank, 2006; Nasca, 2015; Wegener, 2012; Weger, 2018]. However, at the moment, available data suggest that few risk loci have been found for stress-related disorders [Smoller, 2016; Weger, 2018].

These disorders are highly complex and polygenic, and it is likely that each of the identified genetic risk variants makes small contributions to the vulnerable phenotype [Weger, 2018]. Moreover, it is important to note that strong effects associated to genetic alterations have not been found in twin and family studies: for example, the probability that one twin will develop a neuropsychiatric disease (e.g. schizophrenia) when the other twin gets it is only in the range of 40–60% [Lee et al., 2013; Klengel and Binder, 2015; McEwen, 2018].

Indeed, even though a genetic predisposition to susceptibility or resilience has been identified, a great deal of clinical and preclinical research indicates a combined contribution of genetic and environmental factors as a more likely source of individual stress vulnerability.

Nowadays, epigenetic mechanisms are largely recognized as strong candidates for gene-environment interactions that impact stress responsiveness [Franklin, 2012; Klengel and Binder, 2015]. The word "epigenetics" means "above the genome" and considers mechanisms of functional control over the genetic information without changing DNA sequence. These mechanisms include alterations of the chromatin structure and thus accessibility of the DNA to the transcriptional regulators, but also the regulation of transcription and translation by non-coding RNAs [Klengel and Binder, 2015; McEwen, 2018]. A large body of literature demonstrated that epigenetic alterations in the brain are important determinants of stress susceptibility [Nugent et al., 2018; Mallei et al., 2018; Franklin et al., 2012]. Moreover, it is largely accepted that stress itself, particularly during early life, may induce long-lasting epigenetic changes by altering the expression of genes critically involved in epigenetic regulation [Anda et al., 2010; Franklin, 2012; Klengel and Binder, 2015 Romens, 2015; McEwen et al., 2014; Musazzi et al., 2016; McEwen, 2016].

Interestingly, epigenetic alterations seem to be reversible, particularly in certain time windows, through the actuation of health-improving strategies, such as physical exercise

[McEwen et al., 2014]. Recent preclinical studies highlighted that physical exercise and stress exert opposite effects on behavioral functions and brain plasticity, partly by involving the action of brain-derived neurotrophic factor (BDNF) [Ieraci et al., 2015].

1.2 Main systems involved in the stress response

The stress response is a highly adapted and balanced process that involve the interaction of several systems of the body located both in the central nervous system and in the periphery. In particular, the sympatho-adrenomedullary and the hypothalamic-pituitary-adrenocortical (HPA) axes are the two main systems for maintaining or reinstating homeostasis during stress (Figure 1.3) [Ulrich-Lai and Herman, 2009].

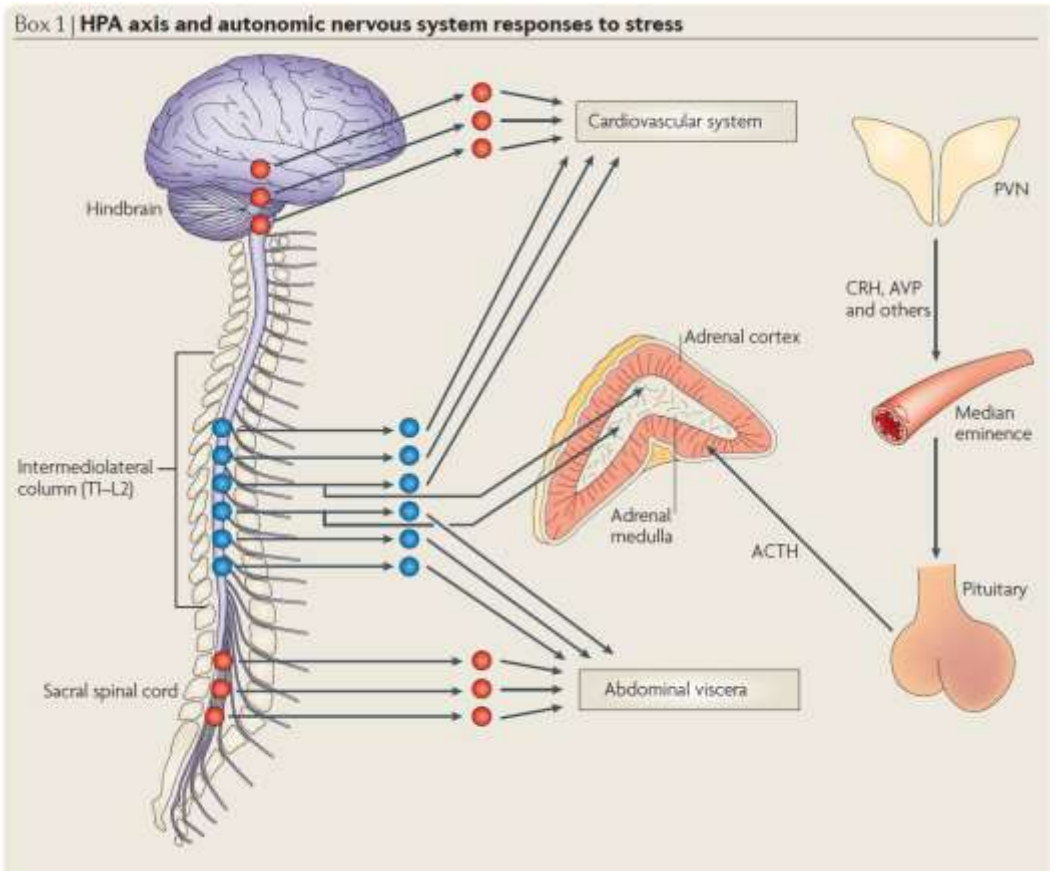

Figure 1.3: Graphical representation of the sympatho-adrenomedullary (see the left-hand side of the figure) and hypothalamic-pituitary-adrenocortical (HPA) (see the right-hand side of the figure) axes. Blue dots: pre- and postganglionic sympathetic neurons; red dots: pre- and postganglionic parasympathetic neurons. ACTH: adrenocorticotropic hormone; CRH: corticotropin-releasing hormone; AVP: arginine vasopressin; PVN: paraventricular nuclei of the hypothalamus [Ulrich-Lai and Herman, 2009].

1.2.1 The sympatho-adrenomedullary axis

The most immediate response to stressor exposure is mediated by the autonomic nervous system (ANS) through the interaction of its sympathetic and parasympathetic arms, which provokes rapid alterations in physiological states through neural innervation of end organs. Preganglionic sympathetic neurons from the intermediolateral cell column of the spinal cord synapse with postganglionic sympathetic neurons, which in turn innervate the smooth muscle of the end organs and chromaffin cells of the adrenal medulla [Charmandari et al., 2005]. The sympathetic response is very fast and transient: it is activated within seconds and it is rapidly turned off by parasympathetic stimulation [Ulrich-Lai and Herman, 2009]. Stress rapidly activates the preganglionic sympathetic neurons that, in turn, stimulate the fast release of the neurohormone epinephrine from adrenal medulla. Although circulating epinephrine cannot cross the blood–brain barrier, the hormone interacts with the central nervous system and stress circuitry by activating vagal nerve β-adrenergic receptors which induce the release of norepinephrine from the nucleus tractus solitarius and locus coeruleus [Kvetnansky et al., 2009; Wong et al., 2012]. The ANS controls a wide range of functions, including cardiovascular, respiratory, gastrointestinal, renal, and endocrine systems [Charmandari et al., 2005; Ulrich-Lai and Herman, 2009]. Hence, the rapid activation of the sympatho-adrenomedullary axis mediates short-term responses that permit the organism to overcome the stressor. These behavioral and physiological mechanisms include changes that increase alertness and provide energy to muscles, as well as alterations that inhibit processes, such as digestion, that can be superfluous during an acute emergency [Wong et al., 2012; Romero and Butler, 2007]. Some of the main alterations mediated by this system are the following: decreasing visceral activity and shutting down digestion; increasing visual acuity; increasing brain blood flow and arousal; increasing gas exchange efficiency in the lungs; breaking down glycogen to release glucose stores; inducing vasodilation in muscles;

inducing vasoconstriction in the periphery; increasing heart rate; and inducing piloerection. This suite of responses, that has immediate effects on increasing the readiness and activity of the animal, comprises the classic Fight-or-Flight response [Romero and Butler, 2007; Cannon and De La Paz, 1911].

1.2.2 The hypothalamus-pituitary-adrenal axis

The hypothalamus-pituitary-adrenal axis (HPA) plays a pivotal role in responding to the environmental demand, by being involved in both stress response and modulation of circadian functions, such as awakening [Clow, 2010; Spencer, 2018]. The regulation of the HPA axis is highly complex. The main components of the HPA axis consist of the medial paraventricular nucleus of the hypothalamus (PVN), the anterior pituitary, and the adrenal cortex. Each of these cell populations secretes a hormonal signal and together form an operational feedforward and feedback closed loop system. The PVN is the key regulatory brain site: it secretes releasing hormones, such as corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), into the portal circulation of the median eminence. These releasing hormones act on the anterior pituitary to promote the secretion of the adrenocorticotropic hormone (ACTH), which in turn acts on the inner adrenal cortex leading to the synthesis and release of peripheral glucocorticoid hormones, mainly cortisol in humans, and corticosterone (CORT) in rodents [Ulrich-Lai and Herman, 2009; Ebner and Singewald, 2017; Spencer et al., 2018]. HPA axis reactivity is regulated to a large extent by feedback mechanisms mediated by glucocorticoids acting on the two brain corticosteroid receptors, the lower affinity glucocorticoid receptor (GR) and the high affinity mineralocorticoid receptor (MR). These receptors can directly inhibit PVN, but since they are localized in several cerebral areas, including hippocampus and amygdala, they can also exert indirect negative feed-back of PVN activity through other neural pathways [Ulrich-Lai

and Herman, 2009; Tasker and Herman, 2011; Ebner and Singewald, 2017]. The complex regulation of HPA axis is schematized in Figure 1.4 [Spencer et al., 2018], below.

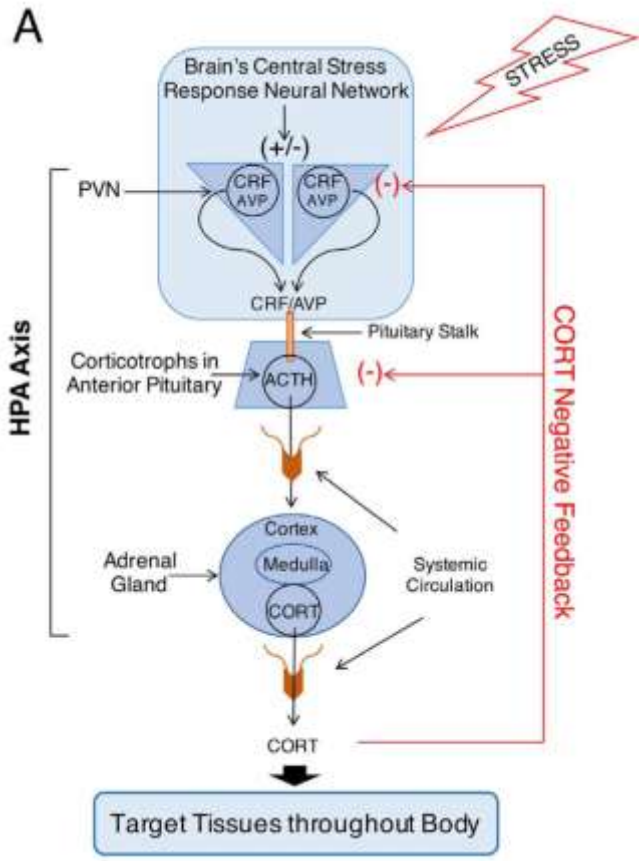

Figure 1.4: The hypothalamic-pituitary-adrenal (HPA) axis and corticosterone (CORT) secretion patterns. See the test above for details. PVN paraventricular nuclei of hypothalamus; ACTH: adrenocorticotropic hormone; CRF: corticotropin releasing factor; AVP: arginine vasopressin [Spencer et al., 2018]

1.2.2.1 Glucocorticoid receptors

As discussed above, glucocorticoids exert their action through the activation of two intracellular receptors, i.e., the high affinity mineralocorticoid receptor (MR) and the lower affinity glucocorticoid receptor (GR), that in the rat is characterized by an affinity even 10-fold lower than the one typical of MR [McEwen et al., 1968; Hollenberg et al., 1985; Arriza et al., 1987; Joels, 2006; Spencer et al., 2018].

This diversity in binding affinity has important consequences for the occupancy of MR and GR during baseline or stress conditions and, consequentially, for the function of the two receptor subtypes in the brain. Based on initial preclinical studies estimating MR and GR

occupancy by CORT in the rat, it was demonstrated that the majority of MR (greater than 90%) is occupied even in basal conditions of hormone secretion, while GR is significantly occupied only at the peak of the circadian cycle or when CORT levels are elevated by acute stress [Spencer et al., 2018]. This occupancy profile suggests that MR may be involved in a tonic (permissive) influence on brain function and that only GR, but not MR, can transduce a phasic change in CORT secretion that occurs with ultradian pulses, acute stress or circadian fluctuation [Reul and de Kloet, 1985; Reul et al., 2000]. On the other hand, a recent study hypothesized a more dynamic fluctuation of MR occupancy and signaling capacity that may vary across tissues for the presence of local CORT buffering factors [Spencer and Deak, 2017; Spencer et al., 2018].

The differential tissue expression of the two receptor subtypes is another important issue in the characterization of the HPA regulation and seems to play a key role in the different functions that MR and GR exert. Indeed, GR is nearly ubiquitous in all tissues and cell types, while MR is more restricted in its distribution, being highly expressed only in the kidney, cardiac tissue, colon, in some immune cells, and in selected brain areas [De Kloet et al., 2000; Arriza et al., 1988]. Importantly, both MR and GR are found throughout the brain. MR is highly expressed in all hippocampal subregions [McEwen et al., 1968; Joëls and de Kloet, 2017], but is less distributed in other cerebral areas, and notably is barely detected in the PVN [Arriza et al., 1988]. On the other hand, GR has high expression in the PVN and is relatively high distributed in most brain regions including the CA1 and dentate gyrus portions of the hippocampus [Herman, 1993; Spencer et al., 2018].

Upon binding to glucocorticoids, MR and GR can exert both genomic and nongenomic effects in the brain through multiple sites and pathways (Figure 1.5) [McEwen et al., 2015]. Classically, MR and GR are both considered nuclear receptors [Spencer et al., 2018]. When not activated by the ligand, MR can be detected in both cytoplasm and nucleus, while GR is

found predominantly in the cytoplasm. Moreover, in the absence of ligand, the two receptors are stabilized in an inhibitory multi-protein complex that includes heat shock protein 90 (hsp90) and FK506 binding protein 51 (FKBP5) [Pratt and Toft, 1997; Binder, 2009]. When bound to the ligand, the receptors dissociate from the complex and accumulate within the nucleus, where they can form homodimers or heterodimers and bind to specific DNA sequences, called glucocorticoid response elements (GREs). Hence, upon binding to GREs, MR and GR can either promote or repress transcription levels of target genes [Gray et al., 2017]. Importantly, glucocorticoid receptors can also interact with other nuclear transcription factors to affect gene expression [Revollo and Cidlowski, 2009; Ratman et al., 2013; Gray et al., 2017].

This genomic mechanism requires a time delay (many minutes to hours) after the increase of serum CORT, but the effects can persist long after CORT secretion has returned to basal levels, likely activating epigenetic mechanisms [Gray et al., 2017; Musazzi et al., 2017; Spencer et al., 2018].

However, as anticipated above, in addition to the classically described transcriptional regulatory process, CORT receptors can also exert rapid (within few minutes) non-genomic alterations. It is claimed that these mechanisms are typical of specific MR and GR localized at membranes, that can act independently of translocation to the nucleus, by modulating intracellular processes and epigenetic changes [Karst et al., 2005; De Kloet, 2008; Groeneweg et al., 2011; Popoli et al., 2012, McEwen et al., 2015]. In the periphery, the functions of rapid effects evoked by glucocorticoids are easily appreciable, since the main effect of glucocorticoid mobilization are significant for supporting the survival of the organism in the minutes during and following an acute stress response (e.g. glucose mobilization, immune suppression) [Tasker et al., 2011; Jiang et al., 2014]. However, the necessity of these same rapid non-genomic mechanisms in the brain are less clear [Tasker et

al., 2011]. Membrane-bound GR and MR are expressed widely throughout the brain and it has been argued that they can rapidly modulate the release of the neurotransmitter glutamate, the activity of the N-methyl-d-aspartate (NMDA) receptor and dendritic spine dynamics [Gray et al., 2017]. In particular, some studies demonstrated that the rapid non-genomic action of CORT receptors is necessary, even though not sufficient (further slower genomic alterations seem to be required), to increase glutamate release after acute stress in rats [Treccani et al., 2014a, see the paragraph below]. Moreover, it has been demonstrated that non-genomic actions mediated by MR and GR affect also other important functions such as modulation of learning and memory processes as well as fast negative feedback on HPA axis activity [De Kloet et al., 2008; Tasker, 2011; Osterlund et al., 2016]. However, other studies hypothesize that the rapid effects of CORT may be mediated by G-protein coupled membrane receptors, whose effects seem to be related to endocannabinoid production [Groeneweg et al., 2012; Popoli et al., 2012 McEwen et al., 2014]. However, these putative receptors still need further characterization [Tasker et al., 2006; Zhang et al., 2012; Spencer, 2018].

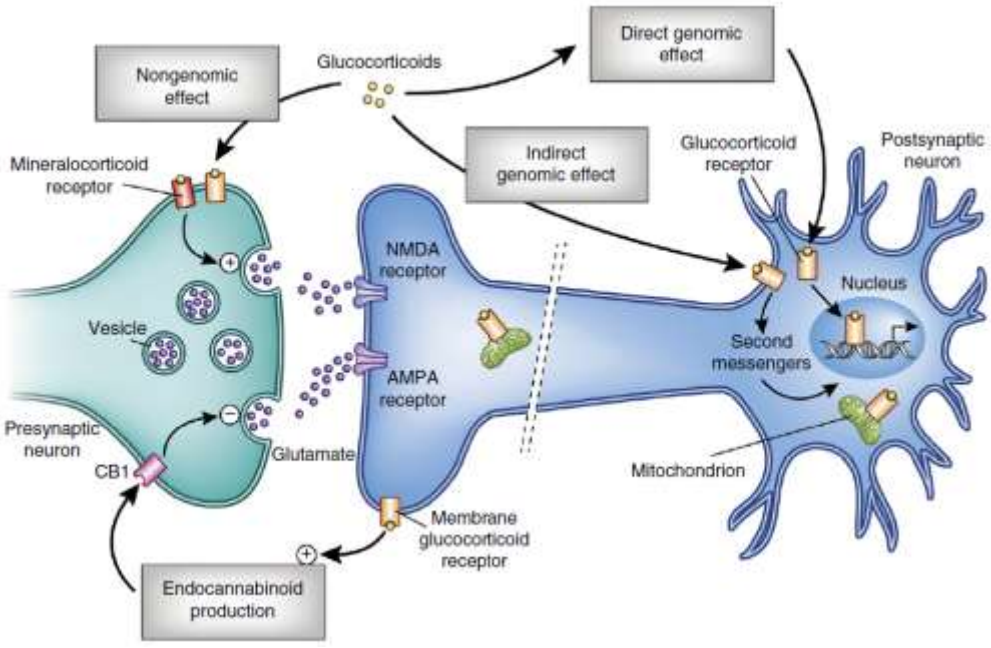

Figure 1.5 Graphical representation of genomic and non-genopic patterns of action showed by glucocorticoids [McEwen, 2015].

1.2.2.2 Glucocorticoids secretion patterns

Glucocorticoids modulate several major cellular processes with regulatory actions that impact all cells and systems of the body, including immunity, inflammation, cell metabolism, energy balance, neural function and response to stressors [Spencer, 2018]. The complex action of glucocorticoid is finely regulated by a rhythmicity of plasma levels, whose functional significance is still poorly understood [Oster et al., 2017].

The endogenous secretion of glucocorticoids is normally characterized by a strong circadian (around the 24 hours) oscillation, with a daily peak that, in most of the vertebrates, rises just before the habitual sleep-wake transition (i.e. in nocturnal animals such as rodents, in the evening, whereas in humans, in the morning) [McEwen et al., 2015; Oster et al., 2017]. The CORT circadian rhythm is normally dependent on the hypothalamic suprachiasmatic nucleus, the body's master biological clock, that controls CORT secretion at both the levels of the PVN and the adrenal gland [Spencer et al.,2018]. Importantly, this rhythm is largely driven by changes in the amplitude and frequency of an hourly ultradian secretion of glucocorticoids, whose origin is still debated [Lightman and Conway-Campbell, 2010; McEwen et al., 2015]. These natural ultradian fluctuations of glucocorticoids levels play pivotal role in brain plasticity [McEwen et al., 2015]. For example, it has been showed that CORT pulsatile action mediates turnover of a subset of synapses in cerebral cortex: an inhibition of the fluctuations with dexamethasone impaired spine turnover [Liston and Gan, 2011]. Notably, these diurnal changes in spine formation and removal resulted to be important for motor learning [Liston et al., 2013]. In addition, a recent study demonstrated that ultradian corticosterone pulses are necessary to ensure a correct balance of glutamatergic transmission and synaptic plasticity [Sarabdjitsingh et al., 2014].

In this context, several studies investigated whether the rhythmicity of glucocorticoids secretion influences how HPA axis modulates stress response. A recent study demonstrated

that maximum stress-induced CORT levels are comparable independently of the time of day of the stressful event, due to a relatively low ceiling of adrenal CORT production rate (Figure 1.6) [Spencer and Deak, 2017; Spencer et al., 2018]. However, it is claimed that stress responsiveness changes in different phases of the ultradian corticosterone secretory profile [Lightman et al., 2010]. Indeed, an elegant study on adrenalectomized rats demonstrated that a brisker HPA axis response (measured as increased levels of ACTH) occurs during the rising phase of plasma CORT, compared with during the falling phase. Moreover, this phase-dependent effect was in line with the behavioral response to the stressor, which was again greater during the rising phase of each CORT pulse [Sarabdjitsingh et al., 2010]. In addition, it has been suggested that rhythmic HPA function is required for the normal initiation and termination of the stress response of ACTH, CORT and other mediators [Akana et al., 1988; McEwen et al., 2015]. Importantly, a dysregulation of the HPA axis has been reported in patients affected by stress-related disorders [Russo et al., 2012; Spencer et al., 2018].

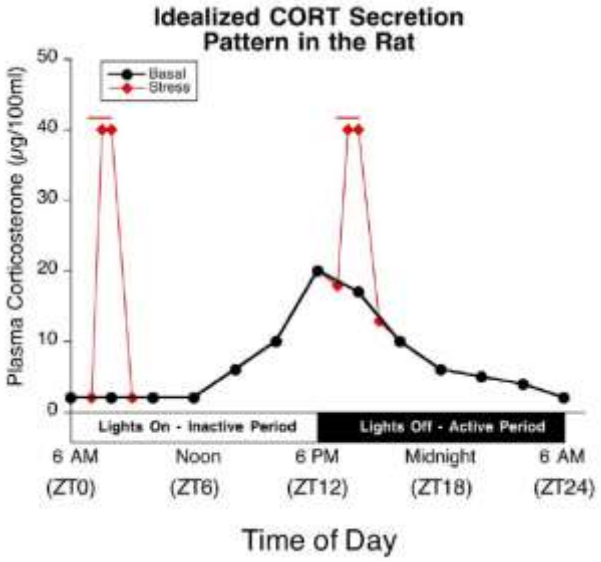

Figure 1.6: Basal corticosterone (CORT) secretion (black line) in the laboratory rat showed a prominent circadian rhythm with a circadian peak around the time of waking (i.e. the onset of the dark phase for nocturnal rats). The red line describes the typical time course and magnitude of increased CORT secretion in response to a short-lasting acute stressor (red horizontal line above each CORT stress peak). Time of day is denoted both in conventional clock time and standardized circadian biology time—zeitgeber time (ZT; hours after onset of light phase) [Spencer et al., 2018].

1.3 Stress in the pathophysiology of neuropsychiatric disorders

Stress and traumatic events exert a dramatic impact on neuronal function and are increasingly recognized as main risk factors for mental disorders, particularly for depression, anxiety disorders, and post-traumatic stress disorder (PTSD) [Popoli et al., 2017]. A large body of clinical neuroimaging studies on depressed patients reported alterations of neuronal architecture in limbic and cortical brain areas [Savitz and Drevets, 2009]. Volume reductions were consistently found in hippocampus (HPC) and prefrontal cortex (PFC), while opposite changes were reported in amygdala [Savitz and Drevets, 2009; Lorenzetti et al. 2009; Koolschijn 2013]. In addition, a reduced density of dendrites and synaptic spines has also been found in the same areas [Kang et al., 2012]. These cerebral regions are recognized to be involved in both the stress response and the cognitive/emotional processing [Duman and Voleti, 2012].

Concomitantly, preclinical studies on rodent subjected to chronic stress showed reduction of synaptic spines, atrophy/remodeling of dendrites and global volumetric reductions, resembling those observed in patients with mood and anxiety disorders [Joëls et al., 2007; Sanacora et al., 2012, see paragraph below]. Moreover, there is the evidence that these regressions can be blocked and even reversed by chronic treatment with antidepressant therapies [Sanacora et al. 2012].

Notably, also one single trauma may determine a life-long pathology, as in the case of PTSD [Hagenaars, 2011]. In parallel, recent preclinical studies demonstrated that a single stressful event can induce sustained alterations in function and neuroarchitecture of PFC in rats [Nava et al., 2017; Musazzi et al., 2017].

Taken together, human and rodent data taken together strongly suggest that these stress-induced alterations are the result of a maladaptive response that plays a pivotal role in the pathophysiology of neuropsychiatric disorders [Popoli et al., 2012; Popoli et al., 2017].

1.3.1 Effects of chronic stress in preclinical studies

Classically, animal models of neuropsychiatric disorders are based on protocols of repeated or chronic stress [Katz et al., 1981; Nestler and Hyman, 2010; McEwen et al., 2014]. Several studies demonstrated that animal subjected to different protocols of chronic stress, gradually develop phenotypical alterations comparable to those observed in patients affected by major depression, including anhedonia [Willner et al., 1992; Berton et al., 2006; Donahue et al., 2014; Naert et al., 2011; Willner, 2017], and alterations in the body weight [Monteiro et al., 2015; Tornese et al., 2019]. Chronic stress induces also pathophysiological changes typically associated to stress-related neuropsychiatric disorders, like dysregulation of the HPA axis [Garcia et al., 2009; Hill et al., 2012; Russo et al., 2012; Tornese et al., 2019], and altered neurotransmitter levels [Hill et al., 2012; Tornese et al., 2019]. In addition, protocols of chronic stress can model functional and behavioral alterations shown by patients affected by neuropsychiatric disorders including cognitive impairment, changes in working memory and fear extinction [Liston et al., 2006; Hains et al., 2009; Eiland et al., 2012; Musazzi et al., 2015].

1.3.1.1 Morphological alterations

As anticipated above, changes in the neuroarchitecture of cerebral areas, involved in cognition and behavioral regulation, were consistently reported in the brain of patients with mood and anxiety disorders and represent key correlates of stress-related pathologies [Popoli et al., 2017].

Classically, HPC provided the gateway into the research about stress and brain structural and functional plasticity [McEwen, 2018], hence a large body of literature describes the morphological alterations induced by chronic stress in this area. Animals subjected to chronic mild stress showed volumetric reduction in the molecular layer of the dentate gyrus, stratum radiatum of CA3, and CA1 [Bessa et al., 2009; Duman and Duman, 2015]. Notably,

these structural changes were reversed by previous treatment with classical antidepressants [Bessa et al., 2009; Morais et al., 2014]. Strikingly, a recent study demonstrated that CMS induces morphological alterations only in animals classified as vulnerable towards stress, based on stress-induced anhedonic phenotype [Tornese et al., 2019]. In addition, dendritic atrophy and loss of spines in hippocampal regions were also found after subjecting animals to chronic unpredictable stress [Qiao et al., 2016], chronic restraint stress [Magarinos et al., 1997; McLaughlin et al., 2007; Gilabert-Juan, 2016], and chronic social defeat [Iñiguez et al., 2016].

Interestingly, it was demonstrated that chronic stress induces similar dendritic remodeling and architectural changes also in cortical regions, including PFC [Radley et al., 2008; Dias-Ferreira et al., 2009; Shu et al., 2017]. Several studies showed that prolonged stress induces dendritic atrophy and reduction of number of synapses [reviewed in Musazzi et al., 2015]. More precisely, chronic stress induces reduction of total dendrites length, together with simplification of the dendritic arbor and loss of spine density [Radley et al., 2004; Radley et al., 2008; Goldwater et al. 2009].

1.3.1.2 The role of glucocorticoids and glutamate in stress-induced morphological alterations

It has been suggested that glucocorticoids could play a major role in the mechanism of synaptic/dendritic remodeling [Popoli et al., 2012]. For instance, it was found that chronic exposure to high levels of CORT decreases spine density and dendritic arbor complexity in HPC as well as mPFC of male rats [Seib et al., 2003, Anderson et al., 2016]. In parallel, it was demonstrated that acute CORT either applicated in vitro or injected in vivo decrease dendritic arborization in hippocampal and cortical neurons [Alfarez et al., 2009; Liston and Gan, 2011].

It has been shown that a major effect of the action of glucocorticoids in HPC and PFC is an enhancement of glutamate release and transmission, which has been hypothesized to be casually related with the retraction of dendrites [McEwen, 2005; Gorman and Docherty, 2010; Popoli et al., 2012; Musazzi et al. 2013].

In addition, the work of Tornese and colleagues demonstrated that a single injection of ketamine, the non-competitive antagonist of the glutamate NMDA receptor, can revert the dendritic retraction found in the CA3 of vulnerable animals subjected to CMS [Tornese et al., 2019].

1.3.2 Effects of acute stress in preclinical studies

As described above, traditional studies approach the field of the physiopathology of stress-related disorders by observing how chronic stress impact neuroarchitecture and function of selected brain areas [Nestler and Hyman, 2010].

However, two main issues suggest that focusing on the effects exerted by chronic stress is not the optimal method to understand how the physiological stress response turn into a maladaptive pathway that may verge towards psychopathology. First of all, it is conceivable that models of chronic stress reproduce only the endpoint of several adaptive changes occurring in the brain and the body during the stress response [Musazzi et al., 2017]. Second, we need to bear in mind that even a single traumatic event can induce long-term pathological consequences in vulnerable individuals [Hagenaars, 2011]. In parallel, it has been recently demonstrated that the effects of a single acute stress are far from being simply acute [for a review, see Musazzi et al., 2017]. In the following paragraph, changes induced by a single inescapable stressor will be discussed.

1.3.2.1 Acute stress induces functional alterations in glutamatergic plasticity

Converging evidence suggested that acute stress is associated with alterations in synaptic plasticity and excitatory transmission in certain forebrain areas, where glutamate neurons predominate [Popoli et al., 2012].

For instance, a recent study suggested that severe stress induces alterations of LTP in HPC and amygdala that are related to a memory impairment [Shoshan et al., 2017]. In this study, rats were exposed to a single severe prolonged footshock (1.5 mA for 10 s) in an inhibitory avoidance apparatus, followed by contextual situational reminders (SRs) of the shock, and changes in LTP and memory impairment were evaluated one month after the stress protocol [Shoshan et al., 2017]. In addition, an elegant work on male rats demonstrated that animals placed in an elevated maze for 30 min showed an impairment of both long-term potentiation (LTP) and pair pulse facilitation (PPF) in HPC, immediately after the stress protocol [Cazakoff and Howland, 2010]. Interestingly, the same study demonstrated that pretreatment of rats exposed to stress with the GR antagonist mifepristone blocked the stress-induced impairment of both PPF and LTP [Cazakoff and Howland, 2010]. In line with these results, the work of Maggio and Segal showed that 15 minutes of forced swim stress rapidly (i.e. within 1 hour) decreases LTP in dorsal HPC by mechanisms involving the activation of GR. On the opposite, the same protocol increases LTP in ventral HPC through MR-dependent mechanisms [Maggio and Segal, 2007].

It was also demonstrated that acute stress increases the amplitude of NMDA- and AMPA-mediated excitatory post-synaptic currents (EPSCs) in PFC [Yuen et al., 2009]. Moreover, patch-clamp recordings of pyramidal neurons in the medial PFC revealed that a single event of inescapable foot-shock stress rapidly reduced PPF, thus suggesting a higher probability of glutamate release [Musazzi et al., 2010].

In addition, it has been widely demonstrated that restraint stress, tail pinch, forced swimming, footshock, and anxiogenic drugs increase the efflux of glutamate as measured by microdialysis in vivo [Lowy et al., 1995]. However, it has been objected that a large portion of amino acid neurotransmitters sampled by microdialysis is of non-neuronal origin, and that the mechanisms whereby acute stress modifies glutamate release are still unclear [Westerink, 1995; Timmerman and Westerink, 1997; Musazzi et al, 2010].

More recent studies measuring release of endogenous neurotransmitters from purified synaptic terminals (synaptosomes) tried to overcome these limitation [Musazzi et al., 2010; Popoli et al., 2012; Treccani et al., 2014a]. This technique allows appreciating glutamate release through precise and selective measurements of endogenous or labelled neurotransmitter release [Raiteri et al., 1974; Raiteri et al., 2000]. The advantage of this method is the avoidance of interferences caused by the interaction of any endogenous ligand with the effectors of the presynaptic machinery, being the thin monolayer of synaptosomes constantly top-down perfused by the medium, resulting in a selective measurement of the glutamate released [Popoli et al. 2012, Figure 1.7].

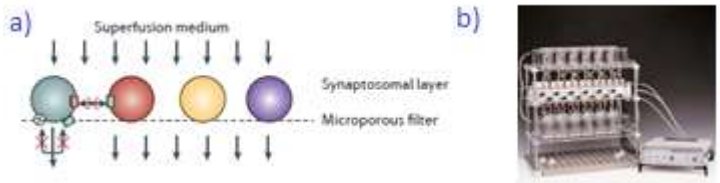

Figure 1.7: The technique of synaptic terminals in superfusion. a) A thin layer of semi-purified or purified synaptosomes is stratified on a microporous filter and a constant up-down superfusion medium is applied to the sample. b) Photography of an apparatus for the superfusion [Adapted from Popoli et al., 2012].

Using this technique, it was demonstrated that one single 40 min session of inescapable footshock (FS)-stress to rats induces rapid enhancement of depolarization-evoked glutamate (not GABA) overflow in prefrontal and frontal cortex (PFC/FC), but not in HPC [Musazzi et al., 2010].

Importantly, it was demonstrated that the alteration of excitatory transmission was mediated by the stress-induced increase of CORT levels, that led to the stimulation of neuronal CORT

receptors, and, in turn, to a rapid accumulation of presynaptic SNARE protein complexes, which mediate glutamate vesicle fusion [Musazzi et al., 2010].

Subsequently, the mechanisms whereby acute stress triggers excitatory transmission and whether these glutamatergic alterations are mediated by local synaptic action of CORT were investigated [Treccani et al., 2014a]. The ex vivo effects of acute stress on glutamate release were compared with those of in vitro application of CORT on synaptosomes. It was found that acute stress increases both the readily releasable pool (RRP, i.e. the ensemble of glutamate vesicles already docked to the membrane and ready to release the neurotransmitter upon stimulus) of glutamate vesicles and depolarization-evoked glutamate release, while application in vitro of corticosterone rapidly increases the RRP, but does not induce glutamate release for up to 20 min.

The increased trafficking of neurotransmitter vesicle towards the synaptic membrane induced by both acute stress and CORT was also confirmed by applying electron microscopy stereology on medial PFC and total internal reflection fluorescence microscopy on synaptosomes, respectively [Treccani et al., 2014a; Khanmohammadi et al., 2017].

Strikingly, Treccani et al. demonstrated also that applying selective CORT receptor inhibitors to the synaptosomes blocks the increase of RRP and vesicle mobilization induced by both acute stress and in vitro CORT application, thus suggesting that the action of acute stress and CORT on vesicle trafficking is mediated by the rapid, non-genomic, action of GR and MR specifically localized on synaptic membranes.

In addition, it was suggested that the molecular mechanisms involved in corticosterone-induced increase of vesicle mobilization require the phosphorylation of synapsin (Syn) I at site 1 (Ser^{9}) in synaptic membranes.

However, since CORT alone was not able to reproduce the stress-induced changes in depolarization-evoked glutamate release in PFC/FC synaptosomes, Treccani et al.,

speculated that rapid (non-genomic) synaptic action of CORT on the RRP size is necessary, but not sufficient, to increase glutamate release/transmission in PFC/FC [Treccani et al., 2014a]. It is likely that slower (> 20 min) genomic effects of the hormone are required to enhance glutamate release [Treccani et al., 2014a; Musazzi et al., 2015]. Indeed, previous studies showed that acute application of CORT on histological PFC sections increases the amplitude of NMDA-mediated EPSCs in layer V of PFC with a mechanism dependent on GR activation, but the amplification of EPSCs currents could be detected 1h after CORT application and was sustained at least for 24h [Yuen et al., 2011].

Interestingly, it was also demonstrated that chronic pretreatment with antidepressants prevents both the enhancement of glutamate release and the increase in the number of docked glutamate vesicles, with a mechanism probably downstream the corticosterone rise [Musazzi et al., 2010; Nava et al., 2015].

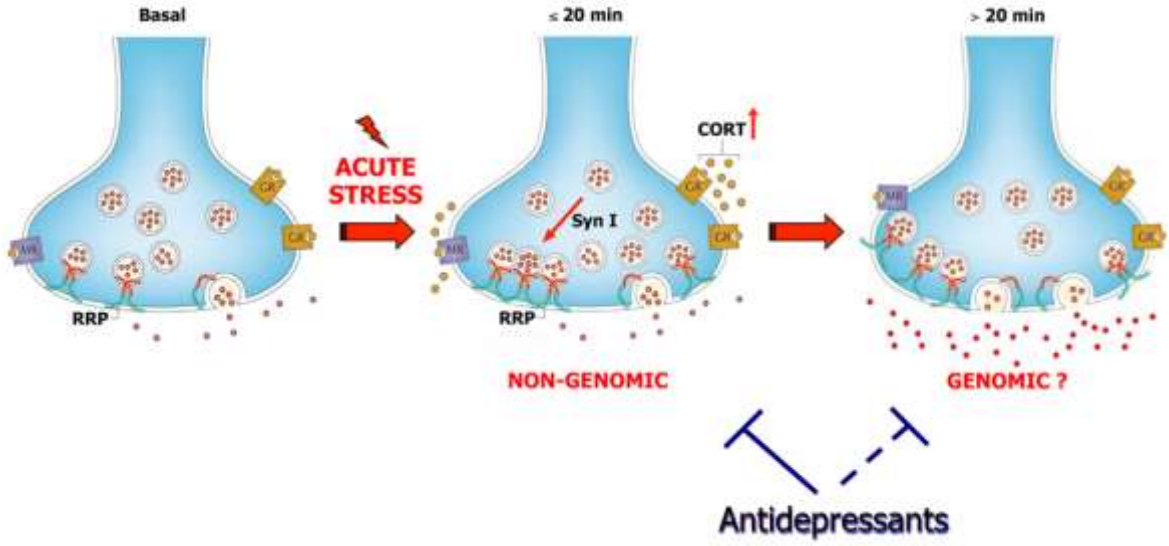

Figure 1.8: Graphical representation of the mechanisms underlying stress-induced increase in glutamate release in prefrontal/frontal (PFC/FC) cortex. The rise of corticosterone (CORT) induced by acute stress causes in synaptic terminals ofPFC/FC a rapid increase of vesicle trafficking into the readily releasable pool (RRP) of glutamate vesicles, with consequent increase of RRP size. This requires binding of CORT to (putatively) membrane-associated synaptic mineralocorticoid/glucocorticoid receptor and non-genomic mechanisms, involving phosphorylation of the presynaptic protein synapsin I. Slower (>20 min), likely genomic effects are required to promote the enhancement of glutamate release and transmission induced by acute stress. Pretreatment with antidepressants dampen the effect of stress on glutamate release [Adapted from Treccani et al.,2014b).

1.3.2.2 Acute stress induces morphological alterations

Even though a large deal of research reported that chronic stress induces long lasting structural remodeling of distinct brain areas (see the paragraph above), few is known about whether acute stress can exert similar dramatic effects on synaptic architecture.

Nonetheless, the analysis of the morphological alterations that a single stressor can exert is likely useful to better understand the adaptive and maladaptive effects of stress response [Musazzi et al., 2017; Musazzi et al., 2018].

While chronic stress induces retraction of apical dendrites and loss of spines in glutamatergic brain areas, including PFC and HPC [Qiao et al., 2016; Tornese et al., 2019], the effects exerted by acute stress are different and region-specific [Kirby et al., 2013].

It has been shown that acute stress increases spine density in hippocampal CA1 of male rats within 24h [Shors et al., 2001]. Interestingly, this result is in line with a previous in vitro study reporting that 1 h of CORT application to HPC slices increases spine density in the same area [Komatsuzaki et al., 2012]. However, opposite effects have been found in CA3, where acute restraint stress induces a rapid decrease of spine density [Chen et al., 2008]. In parallel, Kirby et al. demonstrated that acute stress rapidly enhances neurogenesis in dorsal HPC, without affecting ventral HPC [Kirby et al., 2013].

Recent studies showed that acute stress induces plastic alterations of neuroarchitecture in PFC/FC that are notably rapid [Nava et al., 2015]. It has been shown that both acute restraint stress and acute FS-stress induce ex novo synaptogenesis within 40 min after the stress protocol [Nava et al., 2015]. Interestingly it was demonstrated that both the stress protocols increase the total number of asymmetric (i.e., excitatory) non-perforated synapses in pyramidal neurons of prelimbic PFC layers II/III. However, only FS-stress increased also axo-shaft synapse in the same layer. This specific effect could be related to an activity-dependent synaptogenesis, since FS-stress is more intense and induce higher increase of

CORT levels compared to restraint stress [Nava et al., 2015]. Moreover, these changes were partially blocked by chronic antidepressant pretreatment [Nava et al., 2015]. These results are in line with the dampening action exerted by antidepressants on stress-induced increase of glutamate release and transmission [Musazzi et al., 2010], thus providing a parallel between the modulation of excitatory transmission and changes in structural remodeling [Musazzi et al., 2015].

In addition, striking evidence showed that the rapid morphological alterations induced by acute stress in PFC/FC are sustained over time [Izquierdo et al., 2006; Nava et al., 2017 a]. Indeed, it was demonstrated that acute forced swim stress produces selective shrinkage of medial PFC dendritic tree and that such remodeling can be observed within 72 h [Izquierdo et al., 2006]. In parallel, an elegant study applying time-lapse transcranial two-photon microscopy demonstrated that a single injection of CORT increases spine turnover within several hours in multiple cortical areas [Liston et al., 2011].

More recently, Nava et al. aimed at analyzing time-dependent effects of acute stress on dendritic remodeling within the prelimbic (PL) region of the PFC [Nava et al., 2017a]. In this study, dendritic length and spine density were analyzed 1 day, 7 days, and 14 days after FS-stress. It was demonstrated that acute stress increases spine density at least up to 1 day after the stress protocol. Moreover, significant atrophy of apical dendrites was observed at 1 day after FS-stress and was sustained up to 14 day later [Nava et al., 2017a]. In parallel, it was demonstrated that acute stress exerts significant long-term effects on mRNA levels of Spinophilin, as well as Homer and Shank family genes, deeply involved in spine plasticity [Nava et al., 2017b]. Importantly, long-term effects of acute stress on spine density, dendritic retraction and synaptic plasticity in PFC/FC were prevented at least in part, by chronic pretreatment with desipramine [Nava et al., 2017a; Nava et al., 2017b].

These results added an important piece of knowledge to classic literature since clearly demonstrated that also acute stress, as well as chronic stress, induces dramatic long-term changes to synaptic plasticity and neuronal architecture, at least in cortical regions [Musazzi et al., 2017].

The alteration induced by chronic and acute stress that have been previously described are summarized in the table below (Table 1-1).

NEUROBIOLOGICAL ALTERATIONS INDUCED BY STRESS IN ANIMAL MODELS					
Effects of chronic stress			**Effects of acute stress**		
	Region	Reference		Region	Reference
Morphological alterations			*Morphological alterations*		
Volumetric reduction	HPC	Bessa et al., 2009	Rapid increase of synapses (within 40 min)	PFC	Nava et al., 2015
		Duman and Duman, 2015	Increase of spine density (within 24h)	HPC (CA1)	Shors et al., 2001
		Morais et al., 2014	Decrease of spine density	HPC (CA3)	Chen et al., 2008
		Tornese et al, 2019		PFC	Nava et al., 2017 a
Dendritic atrophy and loss of spines	HPC	Qiao et al., 2016	Enhanced neurogenesis	dorsal HPC	Kirby et al., 2013
		Magarinos et al., 1997	Slower decrease of dendritic arborization	PFC	Izquierdo et al., 2006
		McLaughlin et al., 2007			Nava et al., 2017 a
		Eiland et al., 2012			
		Gilabert-Juan, 2016			
		Iñiguez et al., 2016			
Reduction of total dendrites length,	PFC	Radley et al., 2008			
simplification of the dendritic arbor and		Dias-Ferreira et al., 2009			
loss of spine density		Goldwater et al. 2009			
		Eiland et al., 2012			
		Shu et al., 2017			
Hypertrophy of dendrites	AMY	Eiland et al., 2012			
Functional alterations			*Functional alterations*		
Dysregulation of the HPA axis		Garcia et al., 2009	Alterations (decrease) of LTP	HPC	Maggio and Segal, 2007
		Hill et al., 2012			Cazakoff and Howland, 2010
		Russo et al., 2012;			Shoshan et al., 2017
		Tornese et al., 2019		AMY	Shoshan et al., 2017
Altered neurotransmitter levels	HPC	Ahmad et al., 2010	Impairment of PPF	HPC	Cazakoff and Howland, 2010
		Hill et al., 2012		PFC	Musazzi et al., 2010
		Tornese et al., 2019	Increase of EPSCs	PFC	Yuen et al., 2009
	Striatum	Ahmad et al., 2010	Increase of glutamate release	HPC	Lowy et al., 1995
	FC	Hill et al., 2012		PFC	Musazzi et al., 2010
					Treccani et al., 2014
					Musazzi et al., 2015
			Increase of RRP	PFC	Treccani et al., 2014
Behavioral changes			*Behavioral changes*		
Cognitive impairment		Liston et al., 2006	Impairement of HPC-dependent short-term memory		Shoshan et al., 2017
		Eiland et al., 2012	Enhancement of AMY-dependent memory		Shoshan et al., 2017
Changes in working memory		Hains et al., 2009	Enhancement of working memory, PFC-dependent		Yuen et al., 2011
Other phenotypical alterations			Time-dependent alterations of working memory		Musazzi et al., 2019
Anhedonia		Willner et al., 1992;			
		Berton et al., 2006			
		Donahue et al., 2014			
		Naert et al., 2011			
		Hill et al., 2012			
		Willner, 2017			
Body weight changes		Monteiro et al., 2015			
		Tornese et al., 2019			

Table 1-1: Summary of the main neurobiological alterations induced by chronic stress (blue panel) and acute stress (orange panel). HPC, hippocampus; PFC, prefrontal cortex; FC, frontal cortex; AMY, amygdala.

2 Aims

The stress response is a physiological reaction to environmental changes, orchestrated by the brain, that can be positive and pro-adaptive, or negative and maladaptive, depending on stress intensity/duration and genetic vulnerability [McEwen and Gianaros, 2010; Popoli et al.,2012]. Unfortunately, it is still unknown what mechanisms address the individual responses towards pro-adaptive or maladaptive outcome of stress [Musazzi et al.,2017].

Our previous studies showed that acute footshock stress enhances glutamate release in prefrontal and frontal cortex (PFC/FC), with a mechanism involving rapid, non-genomic enlargement of the readily releasable pool (RRP) of glutamate presynaptic vesicles [Musazzi et al., 2010; Treccani et al., 2014a]. Moreover, we showed that this protocol of acute stress induces long-term atrophy/remodeling of apical dendrites in the same area up to 14 days later, thus suggesting that the consequences of acute stress are far from being simply acute [Nava et al., 2015].

Traditional models of chronic stress reproduce only the endpoint of several adaptive/maladaptive changes occurring in brain and body during the stress response [Musazzi et al.,2017]. On the opposite, analyzing short- and long-term alterations induced by acute stress could be a useful tool to identify the factors that turn the physiological stress response into maladaptive outcomes.

In this context, the main aims of this project are the following:

1- To assess whether acute stress exerts time-dependent alterations on glutamatergic plasticity in PFC/FC.

We evaluated synaptic glutamatergic function, by measuring alterations in glutamate release in PFC/FC. In parallel, we studied the stress-induced changes in the expression of molecular effectors relevant for regulating glutamate release in the same brain area.

2- To compare the effects on glutamate plasticity exerted by acute FS-stress in rats identified as resilient or susceptible to stress on the basis of their behavioral response.

Anhedonia, the lack of interest towards pleasure, is a core symptom of depression and other stress-induced disorders, including post-traumatic stress disorders [Der-Avakian et al., 2012; Nawijn et al., 2015]. Here, we evaluated whether acute stress induces anhedonic phenotype in some of the subjects, thus suggesting a maladaptive outcome of the stress response [Nestler et al., 2010; Christensen et al., 2011; Franklin et al., 2012]. We considered as vulnerable animals showing the anhedonic phenotype 24h after acute stress, while the others were defined as resilient. We then compared functional, molecular and morphological alterations induced by acute stress in the two phenotypes, to identify adaptive and maladaptive trajectories underlying stress response.

Our approach, dissecting rapid and delayed effects of acute stress response, and their adaptive/maladaptive components, could lead to better understand mechanisms that turn stress response from a physiological adaptation into maladaptive alterations. The final aim is to look for the putative effectors of this switch, searching for markers of vulnerability and novel targets for the therapies of neuropsychiatric disorders.

3 Materials and methods

3.1 Animals

All experimental procedures involving animals were performed in accordance with the European Community Council Directive 2010/63/UE and were approved by the Italian legislation on animal experimentation (Decreto Legislativo 26/2014, animal experimentation license N 521/2015-PR). Experiments were performed with adult male Sprague-Dawley rats. Except during the sessions of sucrose intake test , animals were housed two or three per cage, with free access to food and water, and maintained on a 12/12 h light/dark schedule (lights on at 7:00 am), at a controlled temperature ranging between 20°C and 22°C.

3.2 Experimental protocols

To fulfill the aims of the projects, we took advantage of two different experimental schemes. 1- To study the time-dependent functional and molecular alterations induced by acute stress on glutamate system in prefrontal/frontal cortex (PFC/FC), animals were subjected to acute footshock (FS)-stress one week after the arrival in our animal facilities. Hence, we sacrificed animals 40 min, 2h, 6h and 24h after the start of the stress protocol. At these time-points, we measured basal and depolarization-evoked glutamate release together with alterations in the expression of molecular effectors relevant for regulating glutamate release in different fractions of cortical tissue: homogenates, synaptosomes and synaptic membranes (Figure 3.1).

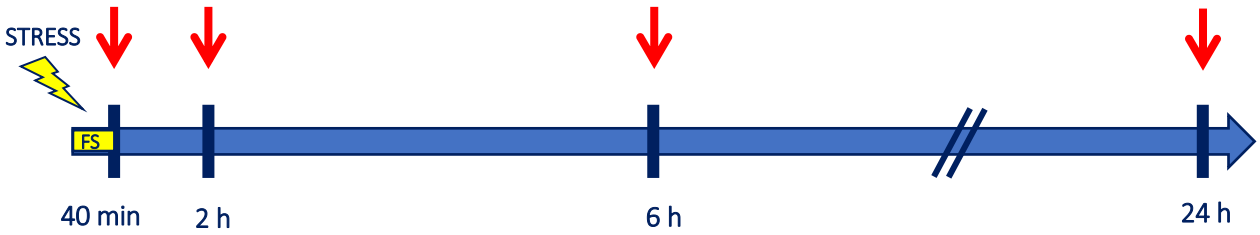

Figure 3.1: Graphic representation of the experimental protocol used to study time-dependent effects of footshock (FS)-stress. Different groups of animals were sacrificed at 40 min, 2h, 6h or 24h after the stress protocol (red arrows). Functional and molecular studies were performed on all the experimental groups. FS: footshock stress.

2- To compare the effects on glutamate plasticity exerted by acute FS-stress in rats identified as resilient or susceptible to stress on the base of their behavioral response, we followed the following scheme. We measured baseline sucrose intake for five weeks, we then subjected 2/3 of the animals to FS-stress. 24h later, we performed a last sucrose intake test for the identification of the phenotypes and, immediately after the end of the test, we sacrificed all the animals (see the paragraph 3.4, "animals classification through sucrose intake test"). Functional, molecular and morphological alterations were assessed in control, resilient and vulnerable animals (Figure 3.2).

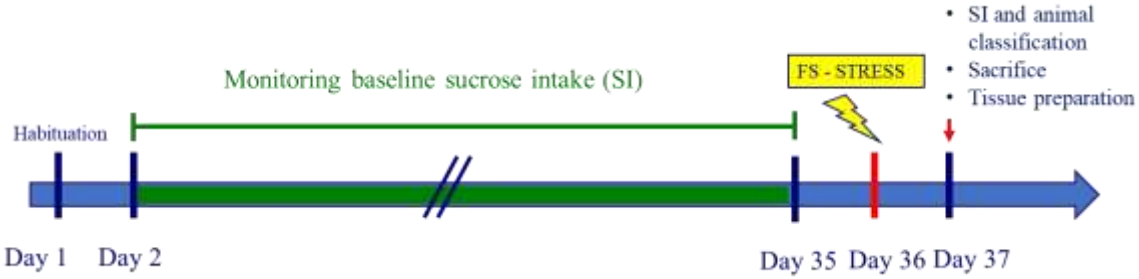

Figure 3.2: Graphic representation of the experimental protocol used to define animals as vulnerable or resilient towards acute stress. Baseline sucrose intake (SI) was measured once a week for five weeks, then randomly animals were subjected to footshock(FS)-stress or left undisturbed in the home cages (controls). 24h after FS-stress, animals were classified as resilient or vulnerable based on the results of a SI test and immediately sacrificed. Tissue were used for functional, molecular and morphological studies.

3.3 Footshock-stress procedure

The footshock (FS)-stress protocol was performed on 275–300g male rats as previously reported [Musazzi et al., 2017, Vollmayr 2001]. Briefly, the FS-stress box was connected to a scrambler controller (LE 100-26, Panlab) that delivers intermittent shocks (0.8 mA) to the metallic floor for 40 min, with 20 min total of actual shock with random single shock and intershock length between 2 and 8 sec. Control animals were left undisturbed in their home cages. Rats were killed by decapitation at different time points after the FS-stress and tissues and blood samples were immediately collected.

3.4 Animals classification through sucrose intake test

To classify animals as resilient/vulnerable towards acute FS-stress, anhedonic phenotype was assessed by performing sucrose intake (SI) test [Christensen et al., 2011]. SI was measured as described in the paper by Christensen (2011) with little changes. The first day (-35d), for two hours, animals were habituated to consume a palatable sucrose solution, by removing water and exposing them to 2 bottles with sucrose solution 1%. From the day after the habituation (-34d), baseline sucrose intake was established giving rats 1 bottle of water and 1 of sucrose 1% for 1 hour, twice a week, for 5 weeks. Bottles were weighted before the start and after the end of the test to measure both the intake of sucrose solution (ml) and sucrose preference, calculated as [(sucrose solution intake (ml)/total liquid intake (ml)]x100 (%). Bottles position in each cage was inverted half an hour after the start of the test. During each session (including the habituation), animals were singly housed, and food deprived. After the baseline SI was established, animals were randomly assigned to FS-stress or left in their home cages (controls). 24h after stress, animals showing at least a 25% within-subject decrease in SI, were considered anhedonic and classified as vulnerable (FS-V), while the others were defined as resilient (FS-R).

In one experiment, we analyzed the time course of the anhedonic phenotype, by measuring SI 6h, 1d, 3d, 7d and 14d after FS-stress (Figure 3.3).

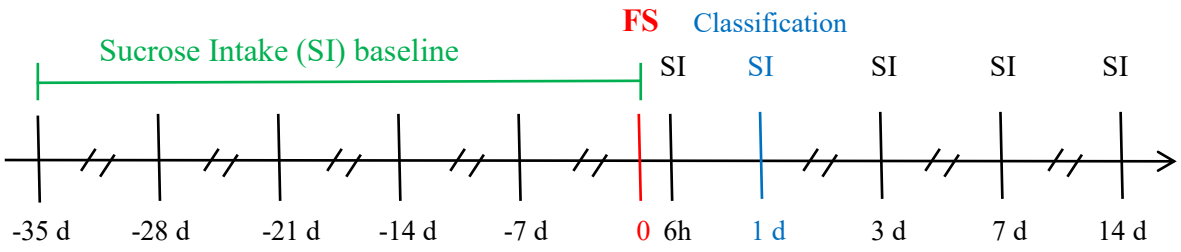

Figure 3.3 The image shows the experimental protocol used to study whether behavioral phenotypes are maintained over time. Sucrose Intake (SI) was measured up to 14 days after footshock (FS)-stress..

3.5 Serum corticosterone assay

Within 1 hour from the sacrifice, blood was centrifuged at room temperature for 20 min at 3000g. The supernatant, separated from blood cells and coagulation factors, was aliquoted and stored at -80 °C. Blood Serum corticosterone levels were measured using a commercial kit (Corticosterone EIA kit, Enzo Life Sciences Inc., Farmingdale, NY) as previously reported [Musazzi et al., 2017].

3.6 Preparation of purified tissue fractions

Nuclei were separated by centrifugation as described previously with modifications [Barbiero et al., 2007]. For neurotransmitter release experiments, purified synaptic terminals (synaptosomes) were freshly prepared by centrifugation on Percoll gradients from PFC/FC, as previously reported [Musazzi et al., 2017]. In some experiments, also glial subcellular particles (gliosomes) were purified by centrifugation on Percoll gradients [Carney et al., 2014] and glutamate release was assessed [Bonanno et al., 2005; Milanese et al., 2009; Milanese et al., 2011]. Synaptosomes and gliosomes were resuspended in physiological medium: 140 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1.2 mM CaCl2, 1.2 mM NaH2PO4, 5 mM NaHCO3, 10 mM glucose, 10 mM HEPES, pH 7.2–7.4. Aliquots of synaptosomes were stored at -80°C for western blotting experiments. The synaptic membrane fraction was prepared by centrifugation from frozen synaptosomes resuspended in lysis buffer: 120 mM NaCl, 20 mM HEPES pH 7.4, 0.1 mM EGTA, 0.1 mM DTT, containing 20 mM NaF, 5 mM Na2PO4, 1 mM Na2VO4, 10µl/ml of phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 2 mg/ml of protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy), as described previously [Musazzi et al., 2017].

3.7 Neurotransmitter release experiments

To measure neurotransmitter release, we took advantage of the method of isolated synaptosomes in superfusion, that allows precise and selective measurements of endogenous or labelled neurotransmitter release [Musazzi et al., 2017]. The advantage of this method is that the thin monolayer of synaptosomes is constantly top-down perfused by the medium, thus preventing any reuptake of released neurotransmitter by autoreceptors and heteroreceptors [Popoli et al., 2012]. In particular, we monitored glutamate release by labeling synaptosomes with [3H]-D-Asp, a non-metabolizable analog of glutamate and a glutamate transporter substrate, widely used to mark the vesicular glutamate pools in release studies [Musazzi et al., 2017; Treccani et al., 2014a]. Fresh synaptosomes from PFC/FC were incubated at 37 °C for 15 min with 0.05 μM [3H]D-aspartate (PerkinElmer, Milano, Italy). To assay depolarization or hypertonic sucrose-evoked releases, either a 90 s pulse with 15 mM KCl or a 15 s pulse with 250 mM sucrose, respectively, were applied. Radioactivity was counted in each released sample collected and in the superfused filters (containing synaptosomes) by liquid scintillation counting. 3H released in each sample was calculated as fractional rate×100 (percentage of the total synaptosomal neurotransmitter content at the beginning of the respective sample collection). In the experiments measuring the action of ketamine on glutamate release, synaptosomes were not radiolabeled, thus enabling us to measure endogenous neurotransmitter release. In this case endogenous glutamate, glycine, Asp and GABA were measured by HPLC analysis of each collected sample. In all the experiments the stimulus-evoked overflow was estimated by subtracting transmitter content of the two 3-min samples (basal outflow) from release evoked in the 6-min sample collected during and after the depolarization pulse (stimulus-evoked release). In some experiments, the same method was applied to measure neurotransmitter release from gliosomes [Milanese et al., 2009; Milanese et al., 2011].

3.8 Western blotting

Western blotting analysis was carried out as previously described [Treccani et al., 2014a] analyzing samples from nuclei, synaptosomes or presynaptic membranes. The BCA protein concentration assay (Sigma-Aldrich, St. Louis, MO, USA) was used for protein quantification. Before electrophoresis, each sample was incubated at 95°C for 5 min. Equal amounts of proteins were applied to SDS polyacrylamide gels (10%) or precast SDS polyacrylamide gels (4–12% Bio-Rad, Hercules, CA, USA), and proteins were electrophoretically transferred to a Hybond-P PVDF Transfer Membrane (GE Healthcare Life Science), for 2 h at a 200 mA. Membranes were blocked for 60min with 5% non-fat dry milk in TBS-T (Tris-buffered saline with 1% Tween-20, Sigma-Aldrich, Milan, Italy). Immunoblotting was carried out with monoclonal antibodies for synapsin I 1:2000 (Synaptic System, Gottingen, Germany), CREB 1:1000 (Cell Signaling), phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204) 1:2000 (Cell Signaling), β-actin 1:20000 (Sigma-Aldrich), and polyclonal antibody for phospho-synapsin I site 1 (Ser9) 1:1000 (Cell Signaling), phospho-CREB (ser133) 1:1000 (Cell Signaling), p44/42 MAPK (Erk1/2) 1:2000 (Cell Signaling), MR 1:500 (Santa Cruz Biotechnology, Dallas, TE, USA), GR 1:500 (Santa Cruz Biotechnology), phospho-GR (ser224, corresponding to phosphorylation on ser211 in humans) 1:1000 (Santa Cruz Biotechnology). All protein bands used were within a linear range, and normalized for β-actin level in the same membrane for loading.

3.9 Analysis of dendritic morphology

Golgi-Cox staining is based on the principle of metallic impregnation of neurons, allowing complete visualization of the neuronal architecture. The main advantage of the method is that the reaction stains only 1-3% of the total neurons present in the sample. This leads to an

optimal visualization of the neurons against the background even in the thick tissue sections that are required to follow the entire path of neuronal projections. The Golgi-Cox reaction is based on the formation of whitish mercuric chloride crystals that are further transformed to the black mercuric sulfide deposit upon alkali treatment [Das et al., 2013]. Immediately after decapitation, left or right hemisphere was randomly chosen for morphological analysis. One hemisphere per animal was rapidly rinsed in PBS and processed for impregnation of individual neurons following the manufacturer's instructions for the rapid Golgi kit (FD Neurotech). In brief, hemispheres were immersed in the impregnation solution, made by mixing equal volumes of Solutions A and B, and stored at room temperature for 14 days in the dark. The impregnation solution was replaced after the first 24 hours of immersion. Then, tissue was transferred into Solution C and stored at room temperature in the dark for 72 hours. The solution was replaced after the first 24 hours of immersion. Subsequently, hemispheres were immersed in ethanol and dry ice and stored at -80°C. Starting from the following day, hemispheres were sliced coronally (200 μm thick sections) on a cryostat. After cutting, sections were left drying over/night. The day after, sections were stained with the staining solutions made mixing equal volumes of Solutions D and E and water, dehydrated through a graded series of ethanols (50%, 75%, 95% and absolute ethanol), cleared in xylene and coverslipped with Eukitt® (Carlo Erba, Cornaredo, Italy). The prelimbic (PL) area of medial prefrontal cortex (mPFC) was identified as previously described [Van Eden and Uylings et al.,1985] using a ×20 objective on a light microscope (Stereo Investigator System). Within PL, layers II–III were identified as previously reported [Van Eden and Uylings et al., 1985]. Using a ×40 objective, pyramidal neurons were identified by dendrites extending into 2 distinct conical arbors, an apical and a basal one (biconical radiation) (Harris and Weinberg, 2012). For each animal within a group, 3–6 neurons were sampled. Z-stacks (80–100 μm; Z-step size 1 μm) of pyramidal neurons with

untruncated branches were acquired using light microscope described above. Z-stacks were processed using the open source Fiji software. Images were then were imported in IMARIS 7.6 (Andor Technology; Belfast, Northern Ireland) and dendrites were semi-manually traced and tridimensionally reconstructed. FilamentTracer processing algorithms centered and determined dendrite diameter and length. Sholl analysis was also performed, in which the center of the soma was used as a reference point.

3.10 Statistical analysis

For the analysis of CORT serum levels, protein expression, glutamate release levels, neuronal dendritic length, one-way ANOVA followed by the Tukey's or Fisher's LSD post-hoc test was used. For Sholl analysis and data from freezing, two-way ANOVA followed by the Tukey's post-hoc test was used. Correlations between 15 mM KCl-evoked [3H]D-aspartate release and CORT serum levels were analyzed by Pearson's correlation. Data are represented as means ± standard error means. Statistical analysis was carried out using GraphPad Prism6 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined when $p < 0,05$.

4 Results

4.1 Rapid and sustained effects of acute stress on glutamate system in PFC/FC

In order to further characterize the response towards acute stress, we studied the early and delayed functional and molecular/cellular alterations induced by a single session of acute FS-stress on glutamatergic plasticity.

4.1.1 FS-stress increases glutamate release up to 24h after the protocol

In recent studies, we found that acute inescapable footshock (FS)-stress rapidly enhances depolarization-evoked glutamate release from prefrontal and frontal cortex (PFC/FC) purified synaptic terminals (synaptosomes) [Treccani et al., 2014a]. In parallel, we showed that the same protocol of acute stress rapidly increases the number of excitatory synapses in PFC/FC and induces alterations in dendritic morphology that are strikingly sustained over time [Nava et al., 2015; Nava et al., 2017 a].

We speculated that sustained enhancement of glutamate release could have a key role in the atrophy/remodeling of dendrites we observed at 24 h and longer times after the acute stress protocol. Thus, we measured changes in glutamate release at different time points (40 min, 2 h, 6 h, 24 h) after start of the FS-stress paradigm [Musazzi et al.,2017].

As expected, CORT serum levels were elevated at the end of the stress protocol (40 min) but returned at basal levels within 2 h as shown in Figure 4.1, a (One-way ANOVA, $F_{4,77}$=98.76, P<0.001, Fisher's LSD post-hoc test: t=40 min, P<0.001). Instead, even though basal glutamate release was not affected by FS-stress (Figure 4.1, b; $F_{4,36}$=0.90), RRP size (measured as glutamate released upon hypertonic sucrose) and depolarization-evoked glutamate release were increased at all times up to 24 h, as shown in Figure 4.1, c and d, respectively (One-way ANOVA followed by Fisher's LSD post-hoc test. RRP: $F_{4,46}$=6.05, P<0.001; t=40 min vs CNT at t=40 min, P<0.05; at t=2h vs CNT, P<0.01; at t=6h and t=24h

vs CNT, P<0.001; depolarization-evoked glutamate release: F4,36=5,31, p<0.01; at t=40 min vs CNT, P<0.05; t=2h vs CNT, P<0.05; at t=6h and t=24h vs CNT, P<0.001). Notably, we measured glutamate release by labeling synaptosomes with [3H]-D-Aspartate (see the paragraph *3.7 Neurotransmitter release experiments* for details).

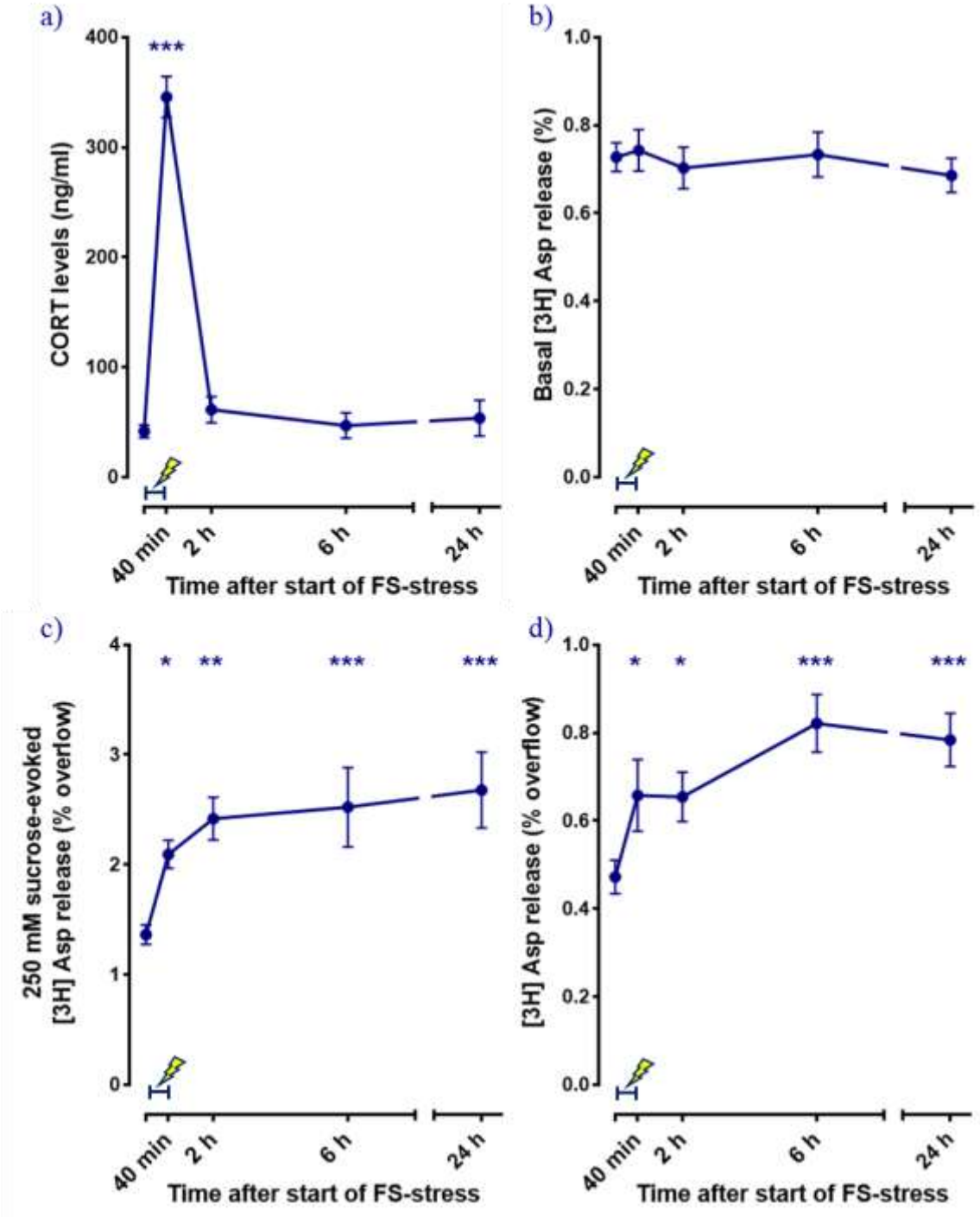

*Figure 4.1: Rapid and delayed (within the first 24h) effects induced by acute footshock (FS)-stress in corticosterone serum levels (a), basal [3H]D-aspartate release (b), readily releasable pool of glutamate vesicles (measured as hypertonic sucrose-evoked [3H]Aspartate (c), and depolarization-evoked [3H]D-aspartate release (d). Data are expressed as % overflow ± s.e.m. One-way ANOVA followed by Fisher's LSD post hoc test. * p< 0,5; ** p< 0,01: *** p< 0,001*

4.1.2 Acute footshock-stress induces time dependent molecular changes in synaptic membranes

In previous studies, we found that the mechanism underlying the stress-induced increase of glutamate release involves CORT rise, the activation of synaptic MR and GR, and rapid, non-genomic enlargement of the readily releasable pool (RRP) of glutamate presynaptic vesicles [Treccani et al., 2014a]. We also demonstrated that the phosphorylation of the presynaptic protein synapsin I (Syn I) at site 1 (Ser^{9}) in presynaptic membranes is required for the increase of the RRP induced by CORT.

Therefore, we assessed whether the rapid and sustained increase of glutamate release induced by acute stress involves time-dependent alterations in the expression and phosphorylation levels of CORT receptors and Syn I in synaptic membranes from PFC/FC. We found that acute FS-stress significantly increases the expression levels of MR (One-way ANOVA $F_{2,24}$=2,69, P<0,001). Fisher's LSD post hoc test showed that MR expression is significantly increased at t=40 min (P<0,01) and t=2h (P<0,05) compared to controls. Moreover, GR expression is significantly triggered by acute stress (One-way ANOVA, $F_{4,40}$=2,70, P< 0,05) with a slower trend compared to MR: Fisher's LSD post hoc test revealed statistical difference at t=6h vs CNT(P<0,01). We also found that the phosphorylation of GR at Ser^{232} is increased (One-way ANOVA, $F_{4,40}$=6,01, P<0,001) at t=2h (P<0,05), t=6h (P<0,01) and t=24h (P<0,001) compared to controls.

Instead, acute stress triggered the phosphorylation levels of Syn I at serine 9 at all the time points up to 24 h (Figure 4.2, c; One-way ANOVA, $F_{4,35}$=8,86, P<0,001. Fisher's LSD post-hoc test: t=40 min vs CNT, P<0,01; t=2h vs CNT, P<0,01; t=6h vs CNT, P<0,01; t=24h vs CNT, P<0,05). No effects of stress were found on the expression of total Syn I (Figure 4.2, c. $F_{4,43}$ =2,48).

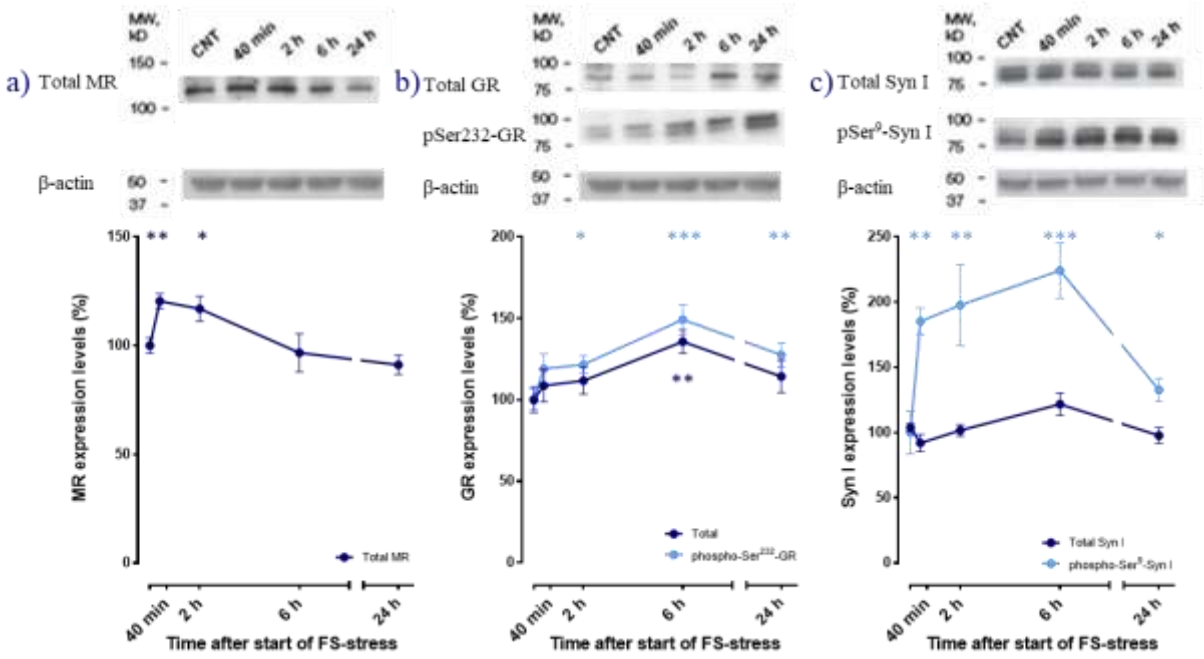

*Figure 4.2: Rapid and delayed (within the first 24h) effects induced by footshock(FS)-stress on the expression of mineralocorticoid receptor (MR, a), the expression of glucocorticoid receptor (GR) and phospho- Ser^{232}GR (b), and the expression and phosphorylation levels at Ser^{9} of synapsin (Syn) I (c). Data are expressed as % of controls ± s.e.m. One-way ANOVA followed by Fisher's LSD post hoc test. * p< 0,5; ** p< 0,01: *** p< 0,001. Insets: representative immunoreactive bands.*

In addition, we demonstrated that the extent of Syn I phosphorylation at Ser^{9} is positively correlated with the size of RRP, in both control and stressed rats (Pearson's correlation analysis, CNT: r =0.5219, P<0.05; FS-stress: r =0.3542, P<0.05) (Figure 4.3, a). No correlation was found for total Syn I level (Figure 4.3, b).

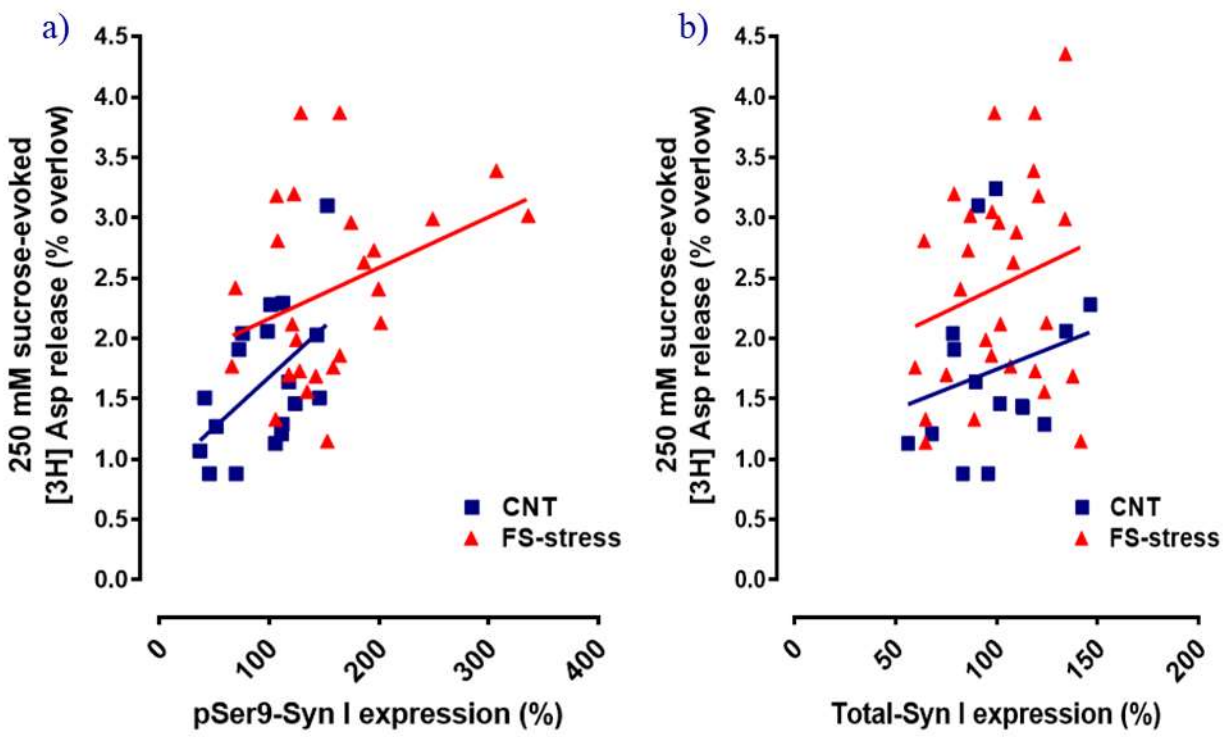

Figure 4.3: Pearson's correlation analysis of 250 mM sucrose-evoked [3H]D-aspartate release and synapsin I phospho-Ser9 (site 1) levels. CNT: r =0.5219, Po0.05; FS-stress: r =0.3542, P<0.05. CNT: controls, FS-stress: animals subjected to footshock-stress protocol; Syn: Synapsin

4.2 Characterization of an animal model of resilience/vulnerability towards acute stress

Since we demonstrated that acute stress, as well as chronic stress, induces sustained effects on glutamatergic plasticity, we aimed at developing a novel model of resilience/vulnerability towards acute stress, in order to further dissect the adaptive/maladaptive components of the stress response.

4.2.1 Validation of the animal model

Since anhedonia is a core symptom of stress-induced neuropsychiatric disorders, including major depression and PTSD, we evaluated whether acute stress induces anhedonic phenotype in some of the animals, thus suggesting a maladaptive outcome of the stress response [Nestler and Hyman, 2010; Christensen et al., 2011; Franklin et al., 2012].

4.2.1.1 Sucrose intake test allowed the classification of resilient and vulnerable phenotypes

We adapted sucrose intake (SI) test, traditionally performed in models of chronic stress [Franklin et al., 2012], to identify anhedonic phenotype induced by acute FS-stress. We measured the baseline SI of each animal once a week for 5 weeks, before subjecting two thirds of the rats to acute inescapable FS-stress. 24h after the stress protocol, animals drinking less than 75% of baseline levels (thus showing anhedonic phenotype) were classified as *vulnerable* (FS-V), while the others were defined *resilient* (FS-R). Even though evaluating the SI of all the stressed animals together no effects of stress were found (Figure 4.4, a. Unpaired Student's t test, $P = 0.08$), it was possible to appreciate that half of the stressed animals showed a significant decreased SI 24h after the FS-stress protocol (Figure 4.4, b. One-way ANOVA, $F_{2,47}=40.97$; Fisher's LSD post hoc test: FS-V vs CNT, $P<0.001$; FS-V vs FS-R, $P<0.001$).

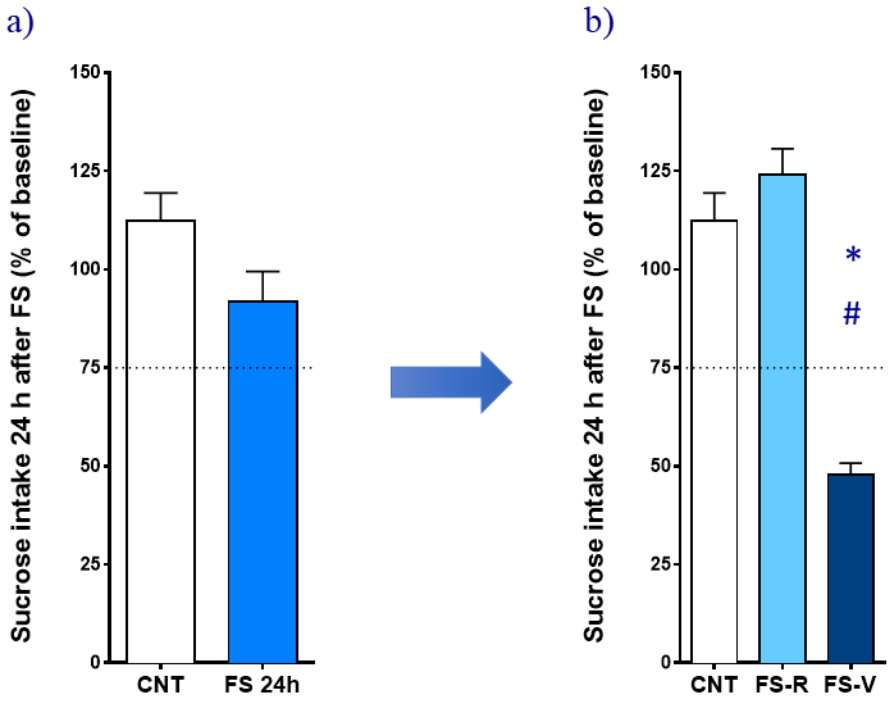

*Figure 4.4: a) sucrose intake (SI) measured 24h hours after footshock (FS)-stress in controls (CNT) and stressed animals (FS 24h). b) SI measured 24h hours after footshock (FS)-stress in animals classified as CNT, resilient (FS-R) and vulnerable (FS-V). Data are expressed as % of baseline SI ± s.e.m. One-way ANOVA followed by Fisher's LSD post-hoc test. * P<0.001 vs CNT; # P<0.001 vs FS-R.*

4.2.1.2 Behavioral differences in the two phenotypes are maintained up to 1 week after FS-stress

In order to further characterize our model and to understand the possible pathophysiological involvement of long-lasting alterations induced by acute stress, we assessed whether the vulnerable/resilient behavioral phenotype was maintained longer than one day after stress. Interestingly, at least in some animals, the anhedonic phenotype was present already 6h after FS-stress, while sucrose intake slowly normalized starting from 72h after the protocol, and hedonic phenotype was completely reestablished in almost all the animals 1 week after the FS-stress (Figure 4.5). Two-ways ANOVA analysis showed a significant effect of both time ($F_{4,164}$=3.708 P<0.01) and phenotype ($F_{2,164}$=19.29, P<0.001), without any effect of the interaction of the two factors. Tukey's post hoc test revealed significant difference between FS-V and CNT only at 24h P<0.001. On the other hand, statistical difference between FS-V and FS-R was maintained at t=24h (P<0.001), t=72h (P<0.01) and t=1week (P<0.01).

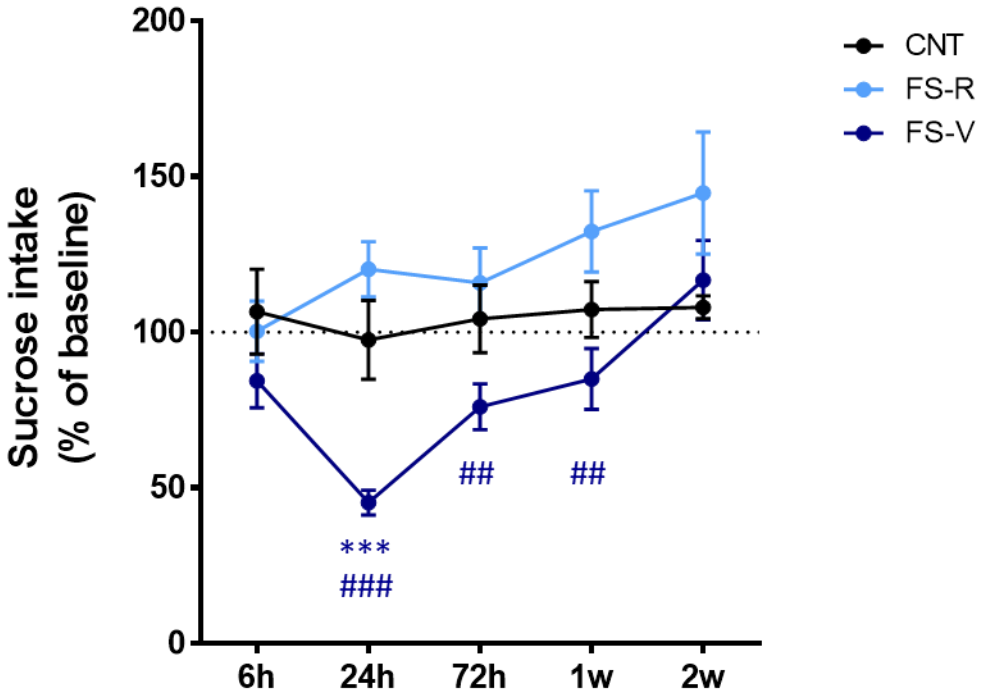

*Figure 4.5: Sucrose intake (SI) measured 24h hours after footshock (FS)-stress in animals classified as CNT, resilient (FS-R) and vulnerable (FS-V). Data are expressed as % of baseline SI ± s.e.m. Two-ways ANOVA followed by Tukey's post hoc test. *** P<0.001 vs CNT; ## P<0.01 vs FS-R; ### P<0.001 vs FS-R.*

4.2.2 FS-stress induces different changes of glutamate release in the PFC/FC of vulnerable and resilient rats

We assessed whether the different behavioral phenotypes were associated with specific functional alterations at the level of presynaptic glutamate release from PFC/FC synaptosomes (measured as % [^{3}H]D-aspartate overflow). Our previous results did not show any effects of acute stress on basal glutamate release at t=24h (see paragraph 4.1.1), however, separating stressed animals based on their behavioral phenotype made possible to highlight that FS-stress increases basal glutamate release selectively in FS-V (Figure 4.6, a. One-way ANOVA, $F_{2,41}$=3.38; Fisher's LSD test: FS-V vs CNT, P<0.05). On the other hand, in line with our previous results, depolarization-evoked glutamate release was increased 24h

after FS-stress in all the stressed animals (Figure 4.6, b. One-way ANOVA, $F_{2,31}$=9.88, P<0.001; Fisher's LSD test: FS-V vs CNT, P<0.001, FS-R vs CNT, P<0.001).

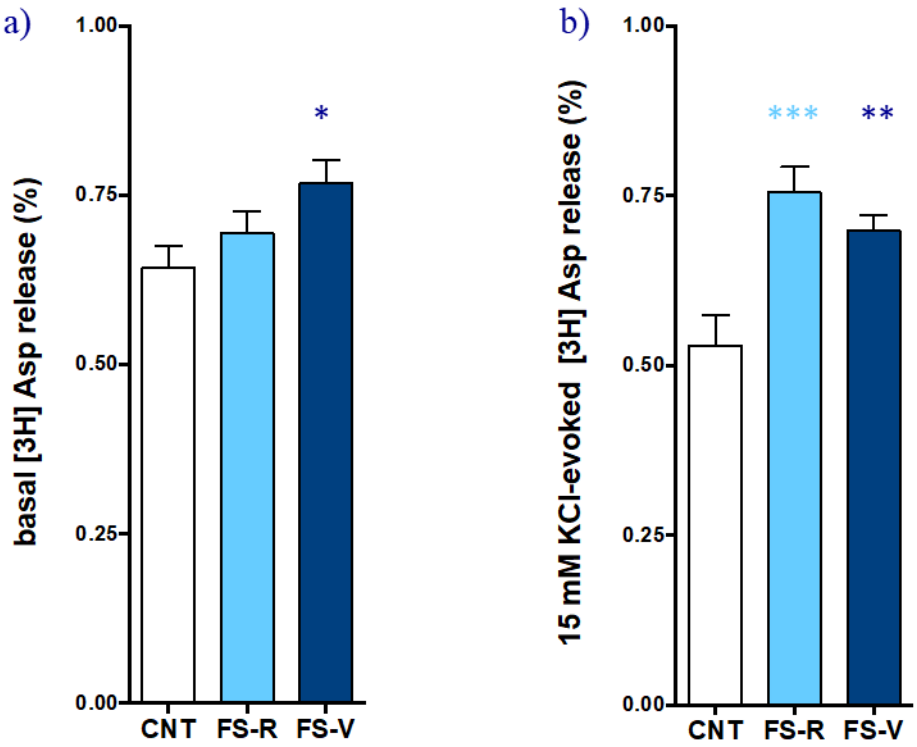

*Figure 4.6: Alterations of basal [3H]D-aspartate release (a) and depolarization-evoked [3H]D-aspartate release (b) induced by footshock (FS)-stress in controls (CNT), resilient (FS-R) and vulnerable (FS-V) animals 24h after the stress protocol. Data are expressed as % overflow ± s.e.m. One-way ANOVA followed by Fisher's LSD post-hoc test. * p< 0,5; ** p< 0,01; *** p< 0,001.*

4.2.3 CORT serum levels are inversely proportional to the increase of glutamate release in FS-R

Our previous analysis of the time-dependent rise of CORT serum levels induced by acute stress showed that the peak of CORT normalizes at t=2h after FS-stress. However, since the definition of the two phenotypes allowed to appreciate differences in basal glutamate release that were not possible to be highlighted before the separation, we measured again CORT serum levels in our model 24h after FS-stress.

We found that in both the groups some of the animals showed very high CORT serum levels still 24h after the stress protocol. However, since the dispersion is large, no statistical

difference was highlighted in either the two groups compared to CNT (Figure 4.7, a. One-way ANOVA, $F_{2,20}$=2.19).

Interestingly, we found that only in FS-R, CORT serum levels are inversely proportional to the increase in depolarization-evoked glutamate release (Figure 4.7, b. Pearson's correlation analysis, FS-R: r = -0,8913, P<0.05).

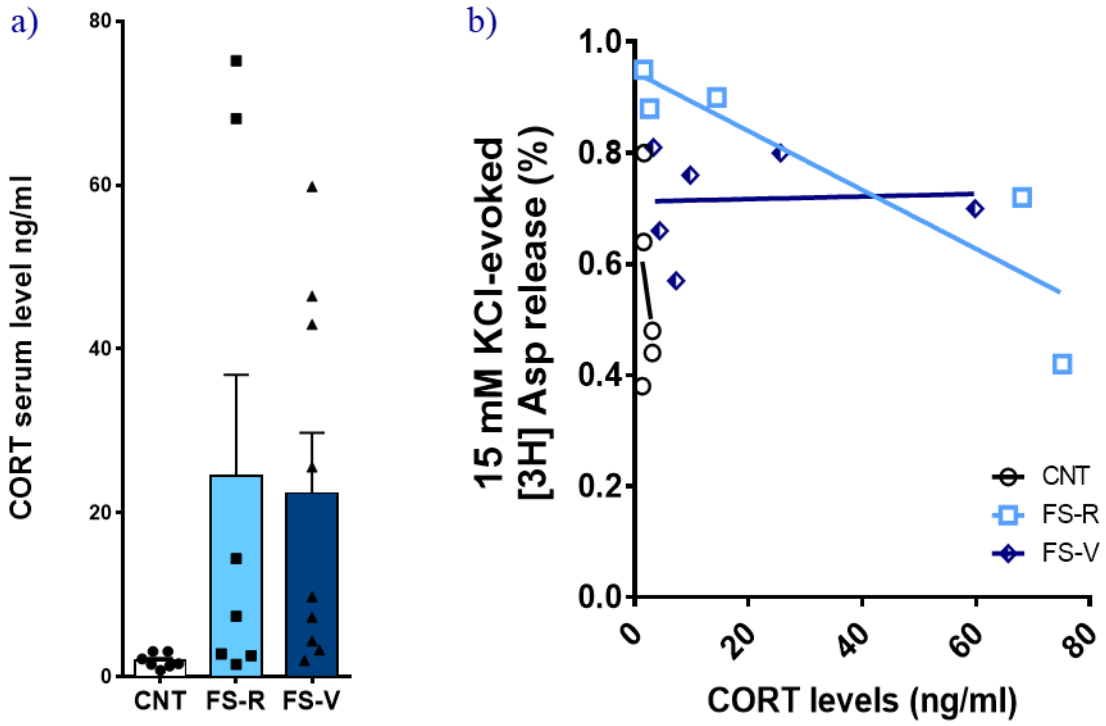

Figure 4.7: a) corticosterone (CORT) serum levels in controls (CNT), resilient (FS-R) and vulnerable (FS-V) animals 24h after footshock (FS)-stress. Data are expressed as % overflow ± s.e.m. b) Pearson's correlation analysis of depolarization-evoked [^{3}H]D-aspartate release and CORT serum levels. FS-R: r = -0,8913, P<0.05.

4.2.4 Acute FS-stress induces phenotype-specific alterations on CORT receptors in PFC/FC

Since we suggested that the regulation of the glutamatergic activation dependent on CORT rise could be different in the two phenotypes, we assessed whether the expression and function of CORT receptors in PFC/FC are different in FS-R and FS-V. The expression levels of MR and the expression and phosphorylation levels of GR at Ser^{232} were measured in nuclear fractions (P1) and synaptic membranes (LP1) from PFC/FC.

We found that 24h after FS-stress the expression of MR in P1 is increased only in FS-R (Figure 4.8, a. One-way ANOVA, $F_{2,24}$=4.35, P<0,05; Fisher's LSD post hoc test: FS-R vs CNT: P<0.01). On the opposite, in the same tissue fraction, the expression of GR was increased only in FS-V (Figure 4.8, b. One-way ANOVA, $F_{2,16}$=4.86, P<0,05; Fisher's LSD post hoc test: FS-V vs CNT: P<0.01; FS-V vs FS-R, P<0.05). No changes in the phosphorylation levels of GR at Ser^{232} were induced by FS-stress in the P1 in either the phenotypes (Figure 4.8, c. One-way ANOVA, $F_{2,26}$=0.95).

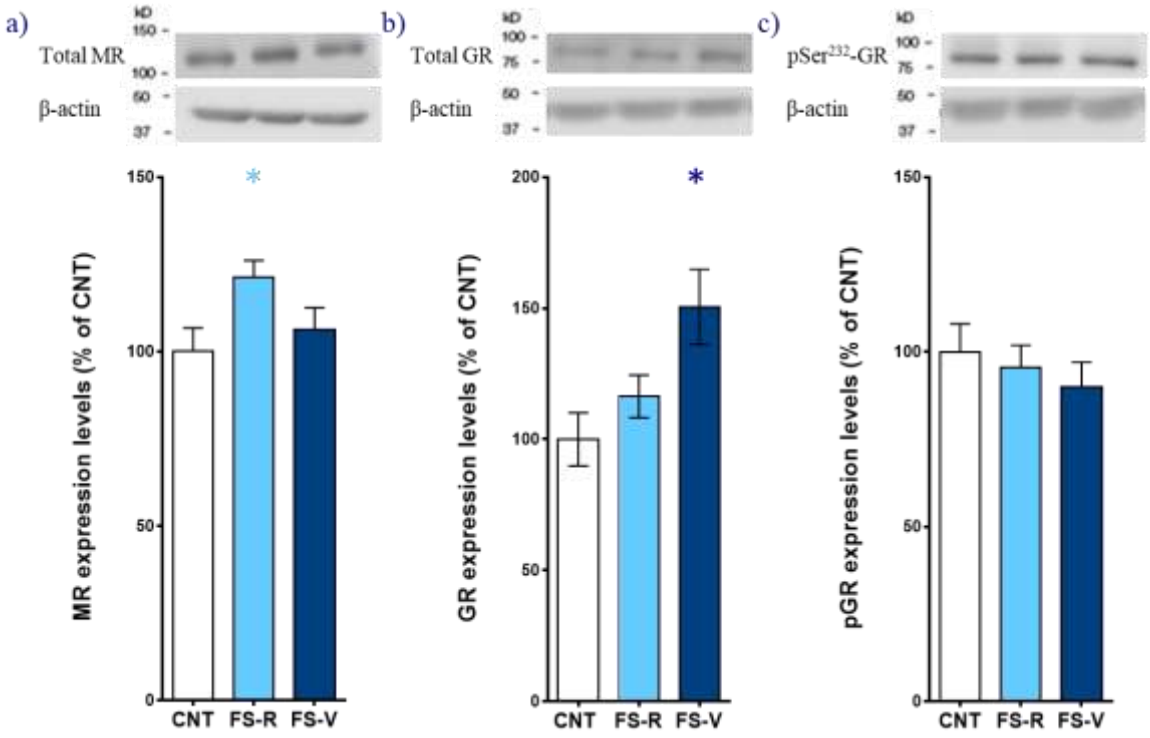

*Figure 4.8: Effects induced by footshock(FS)-stress on the expression of mineralocorticoid receptor (MR, a), glucocorticoid receptor (GR, b) and phospho- Ser^{232}GR (c) in nuclear fractions, 24h after the stress protocol. Data are expressed as % of controls ± s.e.m. One-way ANOVA followed by Fisher's LSD post-hoc test. * p< 0,5 vs CNT. Insets: representative immunoreactive bands.*

In line with the results obtained analyzing the time-dependent effects of acute stress on CORT receptor in LP1 24h after FS-stress, we did not found any effect of stress on the expression and activation of CORT receptors in either the phenotypes (Figure 4.9 a,b and c. One-way ANOVA: MR, $F_{2,33}$=0.93; GR, $F_{2,29}$=0.57; $pSer^{232}$GR, $F_{2,29}$=0.49).

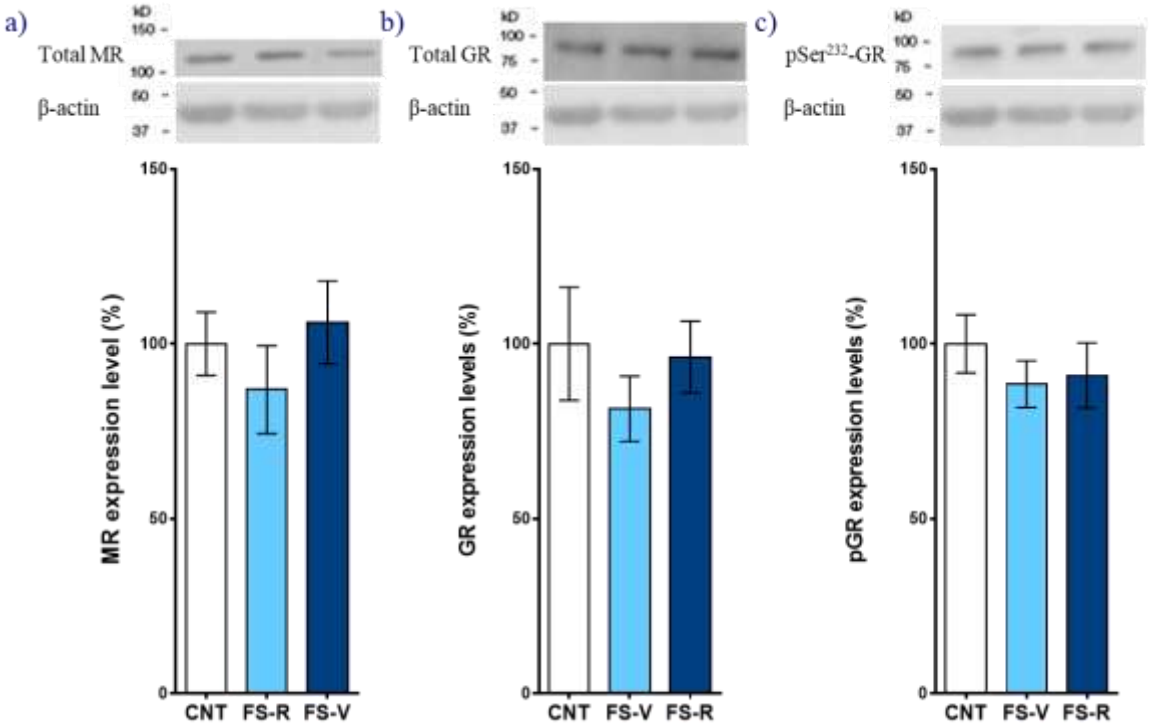

Figure 4.9: Effects induced by footshock(FS)-stress on the expression of mineralocorticoid receptor (MR, a), glucocorticoid receptor (GR, b) and phospho- Ser^{232}GR (c) in synaptic membranes, 24h after the stress protocol. Data are expressed as % of controls ± s.e.m. One-way ANOVA followed by Fisher's LSD post-hoc test. Insets: representative immunoreactive bands.

4.2.5 Acute FS-stress increases the phosphorylation of Syn I in the synaptic membranes from PFC/FC in both the phenotypes

Our previous results demonstrated that 24h after stress the phosphorylation of Syn I at Ser^{9} in synaptic membranes is involved in the sustained increase of glutamate release induced by acute FS-stress. Since our data showed an increase of glutamate release in FS-R and FS-V 24h after FS-stress, we expected to find increased phosphorylation levels of Syn I in both the phenotypes. Our results confirmed this hypothesis. Indeed, we found that even though FS-stress does not increase the levels of total Syn I (Figure 4.10, a. One-way ANOVA, $F_{2,27}$=0.31), its phosphorylation at Ser^{9} is increased in FS-R and FS-V compared to CNT (Figure 4.10, b. One-way ANOVA $F_{2,26}$=5.41, P<0.05. Fisher's LSD post-hoc test: FS-R: P<0.05; FS-V, P<0.01).

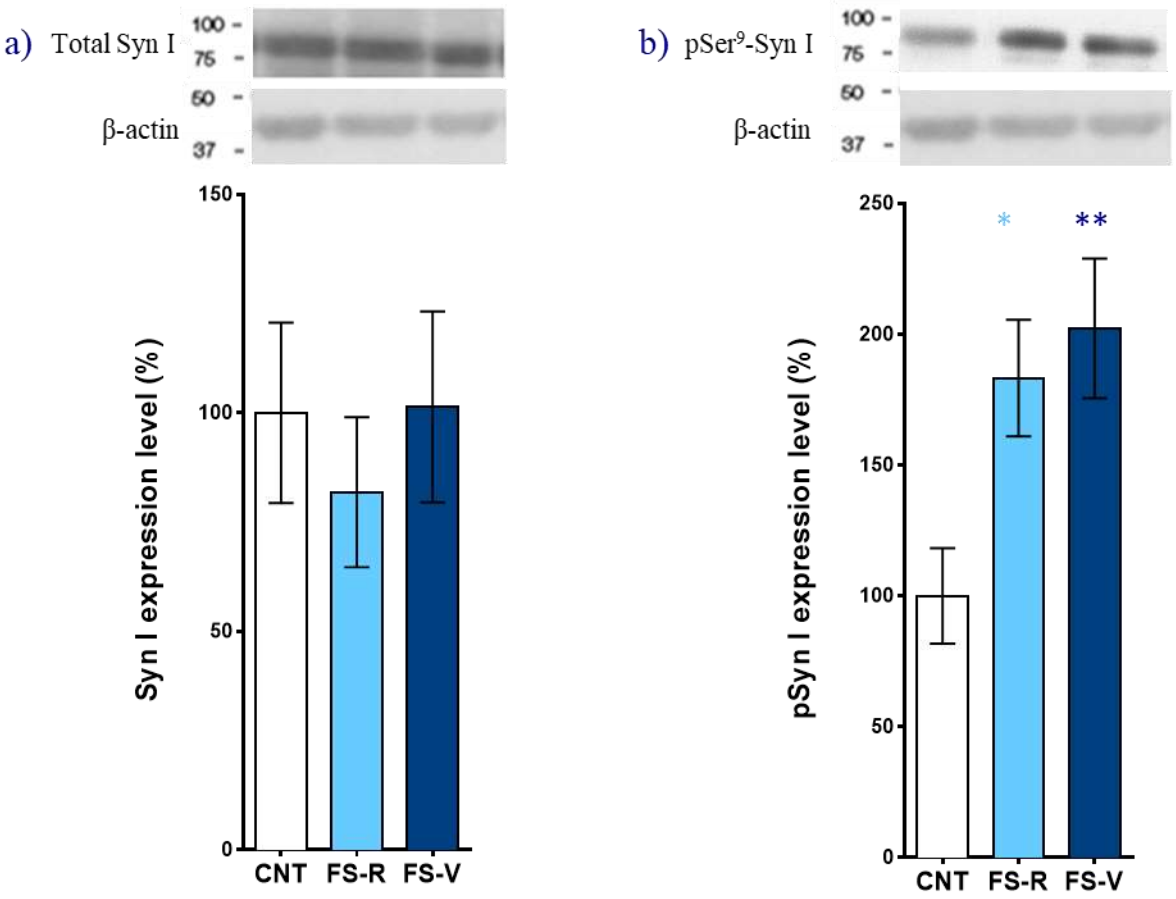

*Figure 4.10: Effects on the expression of Sinapsin (Syn) I (a), and phospho- Ser[9]Syn I (b) in nuclear fractions, induced by footshock(FS)-stress 24h after the stress protocol. Data are expressed as % of controls ± s.e.m. One-way ANOVA followed by Fisher's LSD post-hoc test. * P< 0,5 vs CNT; **P<0.01 vs CNT. Insets: representative immunoreactive bands.*

4.2.6 Analysis of dendritic arborization in prelimbic cortex 24h after FS-stress

Since our previous studies demonstrated that a single session of FS-stress induces rapid and sustained remodeling of apical dendrites in layer II/III of prelimbic cortex [Nava et al., 2015], we investigated whether the behavioral phenotypes could be correlated with specific FS-stress induced morphological alterations in the same area. Taking advantage of Golgi-Cox staining [Nava et al., 2015], preliminary results suggested a stress-induced dendritic retraction in both FS-R and FS-V 24h after stress, however, one-way ANOVA have not yet revealed any statistical significance (Figure 4.11, a. $F_{2,14}$=1.29).

As shown in Figure 4.11, b, two-ways ANOVA of Sholl analysis showed a significant effect of distance ($F_{14, 210}$=16,14, P<0.001) and phenotype ($F_{2,210}$=6.36, P<0.01) on the number of intersections of apical dendrites with the concentric 20 μm-Sholl radii. Fisher's LSD post hoc test revealed that acute FS-stress decreased the number of intersections at central portions of apical dendrites (between 120 μm and 160 μm) in FS-V compared to controls (P<0.05).

As it is possible to appreciate by looking at the graphs reported in Figure 4.11, our results are limited by an exiguous number of observations, particularly concerning the group of CNT and FS-R. It is likely that increasing the number of animals and of neurons analyzed would make our data stronger.

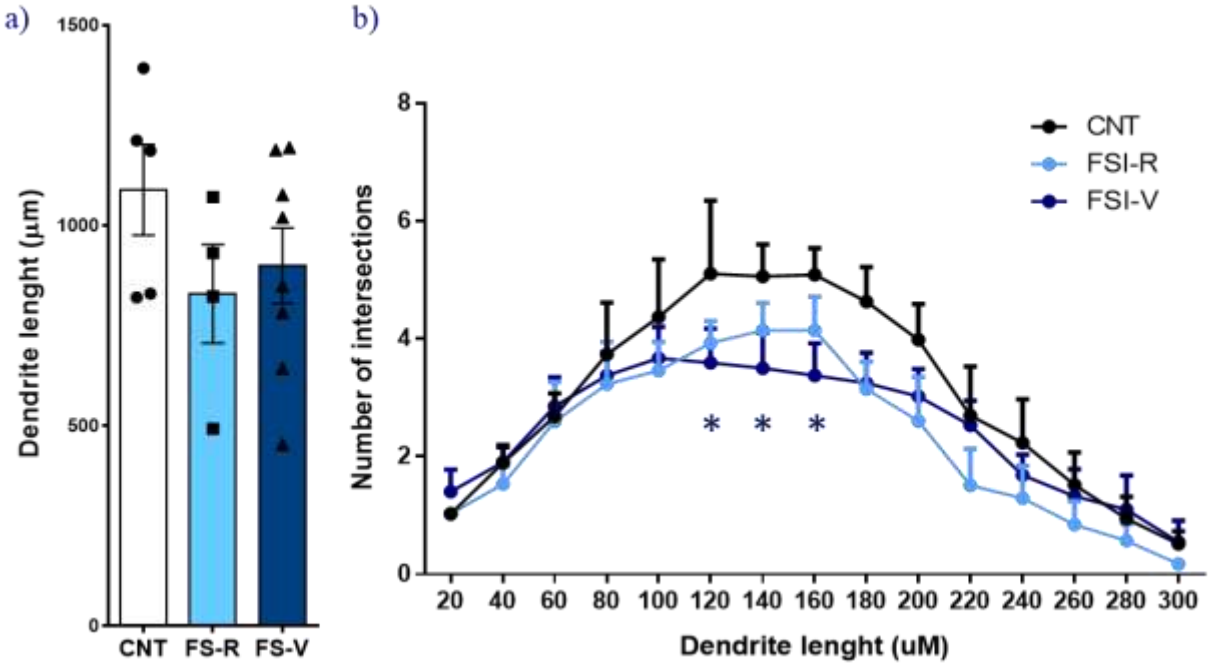

Figure 4.11: a) Analysis of the alterations in dendritic length induced by FS-stress 24h after the protocol in controls (CNT), resilient (FS-R) and vulnerable (FS-V) animals. Data are expressed as means ± s.e.m. b) Sholl analysis of apical dendrites. Two-ways ANOVA of Sholl analysis followed by Fisher's LSD post hoc test. P<0.05 vs CNT.

5 Discussion

5.1 Acute stress is not acute

Most traditional animal models of neuropsychiatric disorders are based on protocols of repeated or chronic stress, although it is known that, in some cases, even a single trauma may be enough to induce a disorder in humans (e.g., PTSD) [Nestler and Hyman, 2010; Hagenaars, 2011; McEwen et al., 2014]. The most consistently found outcomes of chronic stress in rodents are long-term changes in neuroarchitecture in hippocampus (HPC) and prefrontal cortex (PFC), which are accompanied by dysfunctions of the glutamate trasmission [Radley et al., 2008; Popoli et al., 2012; Sanacora et al., 2012].

Strikingly, recent studies demonstrated that also a single session of acute inescapable footshock (FS)-stress induces fast and long-lasting architectural alterations in prefrontal and frontal cortex (PFC/FC), by rapidly increasing the total number of non-perforated synapses in prelimbic PFC (+42%) [Nava et al., 2015], and inducing atrophy/remodeling of apical dendrites in the same area, starting from the day after the stress, and up to 14 days later [Nava et al., 2017 a].

Here, we demonstrated that the same protocol of acute FS-stress induces also functional and molecular/cellular alterations in excitatory synapses in PFC/FC that are both rapid and sustained up to 24h after the stress protocol.

5.1.1 Functional alterations induced by FS-stress are maintained up to 24h after the protocol

In previous studies we have shown that FS-stress rapidly enhances glutamatergic transmission, with a mechanism involving activation of hypothalamic-pituitary-adrenal axis (HPA), increase of corticosterone (CORT) circulating levels and rapid, non-genomic enlargement of the readily releasable pool (RRP) of glutamate presynaptic vesicles [Treccani et al., 2014].

Here, we found that, even though CORT serum levels increased immediately after FS-stress and normalized 2h later, both RRP size (measured as glutamate released upon hypertonic sucrose) and glutamate release evoked by depolarization were increased immediately after the stress paradigm and up to 24 h later. On the other hand, basal glutamate release was unchanged.

Our results are partially in line with the work of Yuen et al. showing that behavioral stressors, through GR activation, induce a prolonged potentiation of NMDAR- and AMPAR-mediated synaptic currents in PFC pyramidal neurons. Indeed, 20min of forced swim stress increased glutamatergic activity starting from 1h after stress, up to 24h later [Yuen et al., 2011]. It could be speculated that the fact that our stress protocol is more prolonged and intense could, at least in part, explain why we reported a more rapid glutamatergic response.

Interestingly, it has been proposed that abnormal enhancement of excitatory transmitter release/transmission is a reason for dendritic atrophy/remodeling in relevant synapses and circuitry, particularly if this is repeated or sustained over time [Popoli et al., 2012; Sanacora et al., 2012; Musazzi et al., 2017]. Since, as reported above, we have previously reported that acute FS-stress induced rapid increase of synapses number and prolonged dendritic remodeling, our current results confirmed this hypothesis [Nava et al., 2015]. A graphic summary of short- and long-term functional and neuroarchitectural effects in PFC synapses after FS-stress is reported below [Figure 5.1; Musazzi et al., 2017; Musazzi et al., 2018].

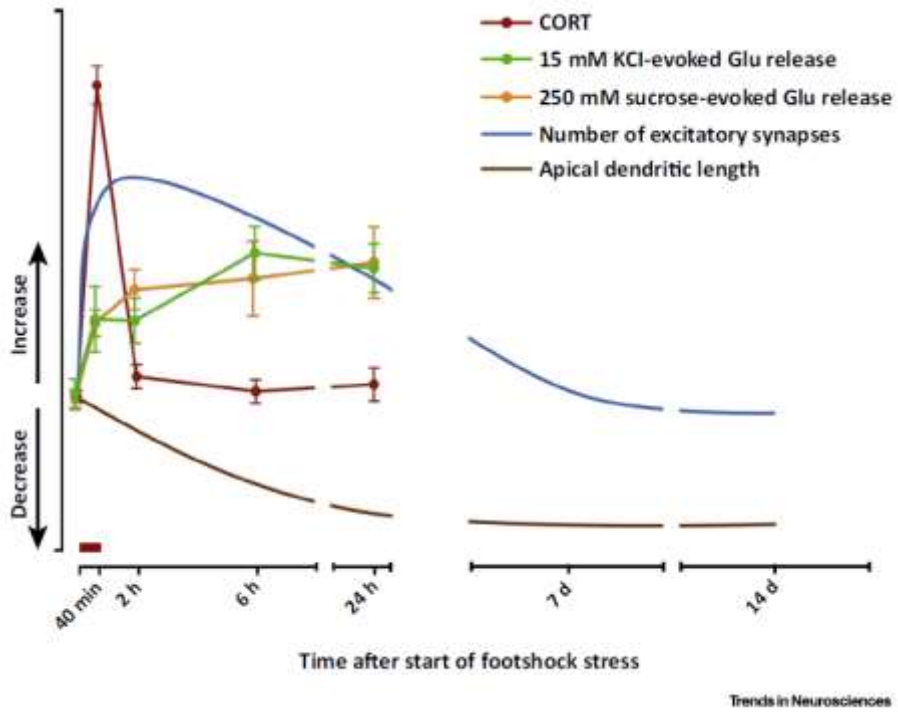

Figure 5.1: The fast and transient increase in corticosterone (CORT) serum levels induced by FS-stress was accompanied by the rapid increase in both readily releasable pool and depolarization-evoked glutamate release in PFC/FC, and the number of small excitatory synapses in layer II/III of prelimbic PFC. The enhancement of glutamate release was sustained for up to 24 h, as well as the increased number of excitatory synapses, which normalized between 24 h and 7 days after FS-stress. Retraction of apical dendrites began 24h after FS-stress and was sustained for up to 14 days [Popoli et al., 2017].

5.1.2 Acute stress induces time-dependent molecular alterations in presynaptic membranes from PFC/FC

In previous studies, we demonstrated that binding to synaptic receptors, CORT induced rapid (non-genomic) enhancement of trafficking of glutamate presynaptic vesicles in perforated synapses, dependent on selective phosphorylation of synapsin (Syn) I at Ser^{9}. This resulted into increased size of the readily releasable pool (RRP) of vesicles, which contributed to the enhancement of glutamate release from synaptic terminals, that likely required slower genomic mechanism [Treccani et al., 2014]. Hence, we assessed whether the sustained increase of glutamate release induced by acute FS-stress was accompanied by time-dependent changes in the expression and activation of CORT synaptic receptors and Syn I.

We found that acute FS-stress exerted different rapid and delayed effects on CORT receptors in synaptic membranes from PFC/FC, with an early and transient increase in MR expression

that is followed by a slower but prolonged increase in the expression and phosphorylation levels of GR. Our results are in line with the hypothesis that MR and GR take part to the regulation of HPA axis and stress response with different kinetics and functions: MR may be involved in a tonic (permissive) influence on brain function, while GR can activate negative feed-back control on peaks in CORT secretion that occurs with ultradian pulses, acute stress or circadian fluctuation [Reul and de Kloet, 1985; Reul et al., 2000; Spencer et al., 2018].

On the other hand, in the study of Yuen et al., only a slow GR activation mediated a delayed increase in glutamatergic activity 1h after acute stress, with no action of MR [Yuen et al., 2011]. However, in that study, it was not possible to appreciate a stress-induced increase in glutamatergic activity up to 1h after the stress protocol [Yuen et al., 2011]. Therefore, it could be conceivable to suggest that the effects of synaptic MR could be mainly involved in regulating early alterations of glutamatergic function. Indeed, high-affinity rapidly activated MRs seem to play a key role in the onset of stress responses, whereas the low-affinity progressively activated GRs are mainly involved in the termination of stress reactions [Yuen et al., 2011].

As already anticipated, since we previously showed that phosphorylation of Syn I at Ser^9 in presynaptic membranes is required for the increase of synaptic vesicle trafficking, in turn responsible for the increase of RRP size and glutamate release, we also measured time-dependent alterations in Syn I expression and phosphorylation levels. As expected, the expression of total Syn I in presynaptic membranes was unchanged, but the phosphorylation of Ser^9 was increased at all times up to 24 h.

Our data confirm the hypothesis that sustained phosphorylation of Syn I after stress is functional to RRP increase and glutamate release enhancement. Indeed, in addition, we

showed that the extent of Syn I phosphorylation at Ser^9 is positively correlated with the size of RRP, in both control and stressed rats, while no correlation was found for total Syn I level. The hypothesis that Syn I is an important component in the regulation of neurotransmitter release in the mammalian nervous system was already known several years ago [Nichols et al., 1992]. However, the mechanisms underlying its action are still unclear [Musazzi et al., 2017]. Ser^9 of Syn I is a consensus site for phosphorylation by cAMP-dependent protein kinase or calcium/calmodulin-dependent protein kinase I [Cesca et al., 2010], but different studies hypothesize the involvement of other kinases in regulating the action of Syn I on glutamate release [Jovanovic et al., 2001; Leon et al., 2008]. Moreover, it has been suggested that a portion of total Syn I does not dissociate from synaptic vesicles, and remains associated with the RRP [Cesca et al., 2010]. It would be interesting to further study how Syn I is involved in the effect of stress on the presynaptic machinery.

5.2 Dynamic dissection of acute stress: a novel approach to investigate the stress response

Since our previous results suggested that the consequences of acute inescapable stress in PFC are far from being simply acute, we postulated that dissecting the short- and long-term outcome of acute stress could be more useful than traditional studies with chronic stress, to understand which are the factors involving in turning the physiological stress response into maladaptive alterations.

5.2.1 Validation of the animal model of stress resilience/vulnerability towards acute stress

Anhedonia, the lack of interest towards something pleasant, is a core symptom of major depression and other neuropsychiatric disorders. Classical studies utilize sucrose intake (SI)

test to assess the development of anhedonic phenotype induced by chronic stress. Here, we set up a novel paradigm to assess whether acute FS-stress alters natural sucrose intake in some animals, revealing stress-induced development of anhedonic phenotype and thus suggesting a maladaptive outcome of the stress response.

It was possible to appreciate that half of the stressed animals showed a significant decreased SI (less than the 75% of the usual SI measured once a week, for 5 weeks, before FS-stress) 24h after the FS-stress protocol. Anhedonic animals, were classified as vulnerable, while the others were defined as resilient (FS-R).

In addition, we demonstrated that these two groups of animals showed different behavioral phenotypes at least up to 1 week after the FS-stress protocol, thus suggesting that the model could possible reflect long-lasting alterations induced by acute stress.

5.2.2 Functional alterations induced by FS-stress in vulnerable/resilient animals

Our priority was then assessing whether the different phenotypes were characterized by specific functional alterations in glutamate release (measured as % [^{3}H]D-aspartate overflow).

Interestingly, we found that acute FS-stress increased basal glutamate release only in FS-V rats, while enhanced depolarization-evoked glutamate release in both FS-R and FS-V rats compared to controls. In addition, only in FS-R, CORT serum levels were inversely proportional to the increase of glutamate release.

Our previous analysis of the time-dependent functional alterations induced by FS-stress, while revealing a sustained increase of depolarization-evoked glutamate release, did not show any effect of acute stress on basal glutamate release at t=24h. However, our classification allowed us to highlight a fine alteration that could be involved in a maladaptive stress response.

Basal glutamate release is a measure of resting (i.e. spontaneous) glutamate levels, discovered in the 1950s [Fatt and Katz, 1952]. Spontaneous synaptic vesicle fusion is a salient feature of all synapses, however, mechanisms regulating spontaneous exocytosis remain poorly understood [Zucker, 2005; Atasoy, 2008]. It is claimed that spontaneous release of glutamate is important for maturation and stability of synaptic networks, controlling spike timing in the brain, maintaining synaptic strength and regulating postsynaptic responsiveness during homeostatic synaptic plasticity [Kavalali et al., 2006; Vyleta et al., 2011; Kononenko and Haucke, 2012]. Interestingly, it has also been proposed that spontaneous release (eletctrophysiologically, also defined as miniature synaptic events, or minis) is involved in the acute regulation of dendritic protein synthesis in neurons [Stutton et al., 2006]. Dendrites in which both action potentials and minis were blocked showed enhanced protein synthesis, suggesting that minis inhibit dendritic translation. When minis were acutely blocked or stimulated, an immediate increase or decrease, respectively, in dendritic translation was observed [Stutton et al., 2004]. This theory could suggest that a wrong control of basal glutamatergic activity could be involved in maladaptive alterations induced by stress on dendritic morphology.

Even though the difference between vulnerable and resilient animals are not strong enough to reach statistical significance, our result could suggest that, in vulnerable animals, mechanisms regulating glutamate homeostasis fail in correctly reestablishing the glutamatergic activity, either because the timing of the response is incorrect or because the inhibitory response induced by hyperactivation of spontaneous release is overactivated.

In addition, although more studies would be required, our data showing that CORT serum levels were inversely proportional to the increase of glutamate release only in FS-R could support the hypothesis of a different regulation of the response towards stress in the two phenotypes. Indeed, it would be worth assessing whether resilient animals, and not

vulnerable subjects, can activate a proper feedback mechanism that modulates the response towards stress-induced CORT increase.

5.2.3 Acute FS-stress induces phenotype-specific alterations on CORT receptors in PFC/FC

To further understand whether the regulation of the glutamatergic activation dependent on CORT rise was different in the two phenotypes, we assessed the expression of CORT receptors in both synaptic membranes (LP1) and nuclei (P1).

As expected, analyzing LP1 purified from PFC/FC from animals sacrificed 24h after FS-stress, we did not find any effect of stress on the expression and activation of CORT receptors in either the phenotypes. Indeed, we previously proposed that the action of synaptic CORT receptor is likely non genomic and it is conceivable that changes in their expression could be appreciable only at earlier times after FS-stress. Consistently, the time course of FS-stress induced alterations of CORT receptors showed that expression levels of both MR and GR were already normalized 24h after the stress protocol (see the paragraphs 4.1.2 and 5.1.2).

On the other hand, we have previously demonstrated that acute stress-induced increase in glutamate release involved also slower genomic mechanisms [Treccani et al., 2014a]. Since, as discussed above (paragraph 1.2.2.1), MR and GR act as nuclear transcription factors, we looked at alterations of their expression and activity in the nuclei.

Interestingly, we found that 24h after FS-stress the expression of MR in P1 is increased only in FS-R. On the opposite, in the same tissue fraction, the expression of GR was increased only in FS-V. Therefore, it is conceivable that this data added another piece of evidence on the hypothesis that FS-R and FS-V showed differences in the activation of mechanisms regulating the response towards acute stress.

5.2.4 Acute FS-stress increases the phosphorylation of Syn I in the synaptic membranes from PFC/FC in both the phenotypes

We have previously demonstrated that the phosphorylation of Syn I at Ser^9 in LP1 is required to the rapid increase in glutamate release and readily releasable pool induced by stress [Treccani et al., 2014a], and suggested that the same phosphorylation is also functional to the sustained increase of RRP up to 24h after FS-stress (see paragraph 4.1.2). Consistently, here we showed that increased glutamate release induced by FS-stress is accompanied by an increment of phosphorylation levels of Syn I at Ser^9 is in both FS-R and FS-V compared to CNT. Moreover, as expected FS-stress did not increase the levels of total Syn I.

5.2.5 Dendritic arborization in prelimbic cortex 24h after FS-stress

Alterations in dendritic morphology and neuronal architecture have been related to the maladaptive effects of stress [Popoli et al., 2012]. Our previous studies demonstrated that a single episode of acute FS-stress, as well as chronic stress, can induce dendritic retraction in PFC up to 14 days after the stress protocol [Nava et al., 2015]. Here, our preliminary results suggested a stress-induced dendritic retraction in both FS-R and FS-V 24h after FS, however, the difference compared to control animals are not statistically significant due to a limited number of observations. Sholl analysis suggested that dendritic retraction is more evident in FS-V compared to CNT and is localized in central portions of dendrites. It would be interesting to increase the number of neurons analyzed to make our results stronger.

The presence of dendritic remodeling in FS-R, as well as FS-V, is not surprising. Indeed, changes in neuroarchitecture are part of a physiological remodeling mechanism in the adult brain, that is required for adaptation and learning [McEwen et al., 2015]. It is conceivable to hypothesize that early after stress, dendritic retraction could be an adaptive mechanism to protect neurons from the increase of glutamate levels induced by stress [Popoli et al., 2012; McEwen et al., 2015]. Later on, in healthy subjects, architecture changes could be reversed

by adaptive mechanism, while in vulnerable subjects, dendritic atrophy is maintained: resilience and recovery from stress-induced changes in neural architecture after stress may be thought of as an active process that implies ongoing adaptive plasticity [McvEwen et al., 2015b]. To confirm this hypothesis, it would be interesting to study morphological alterations in the two phenotypes at other time points after FS-stress, such as after 7 and 14 days, i.e. when our previous study showed that acute stress can still induce dendritic retraction [Nava et al., 2015].

6 Conclusions

Traditionally, animal models of neuropsychiatric disorders are based on chronic stress protocols. However, chronic stress models reproduce only the endpoint of several adaptive changes occurring in the brain and the body during the stress response [Musazzi et al., 2017]. In the present work we showed, instead, that also acute stress, as well as chronic stress, induces rapid and sustained functional, molecular and morphological alterations in excitatory synapses in prefrontal/frontal cortex. Therefore, we proposed a novel animal model to identify resilience/vulnerability towards acute stress, suggesting that dissecting the short- and long-term outcome of acute stress could be more useful than traditional studies with chronic stress to understand which are the factors that turn the physiological stress response into maladaptive alterations. Interestingly, we found that acute stress induced phenotype-specific functional and molecular alterations in glutamatergic synapses, thus confirming our hypothesis. We believe that our approach could help to understand the determinants of a proadaptive versus maladaptive trajectory of stress response.

6.1 Future directions

We claim that it would be interesting to further characterize our model, particularly analyzing long-term morphological alterations induced by acute stress in resilient and vulnerable animals. In particular, we are planning to evaluate phenotype-specific alterations in dendritic arborization induced by acute stress 1 and 2 weeks after FS-stress, the same time points analyzed in our previous studies [Nava et al., 2017].

Interestingly, we recently demonstrated that ketamine (KET), an NMDA receptor antagonist that at low doses showed antidepressant properties [Duman, 2014], can modulate synaptic plasticity and the response towards chronic stress and acute FS-stress [Tornese et al., 2019; Musazzi, Sala et al., manuscript in preparation]. We are going to tackle the hypothesis that KET could revert the alterations induced by acute FS-stress in vulnerable rats.

Our model could be a useful tool for studying the role of novel therapeutic strategies for treating stress-related neuropsychiatric disorders, including PTSD.

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