University of Genoa

Pharmacy Department

Pharmacology and Toxicology Section

PhD Course in Experimental Medicine

Curriculum of Pharmacology and Toxicology

(XXXIV cycle)

Acute stress induces short- and long-lasting modifications of glutamate transmission in the prefrontal cortex at pre- and peri-synaptic compartments

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ABSTRACT

Physiological stress promotes adaptive plasticity, but the mechanisms responsible for maladaptation in the presence of an excessive or dysregulated response still remain unknown. Exposure to an acute inescapable foot shock (FS) stress protocol can induce both rapid and sustained changes in synaptic function and neuroarchitecture, suggesting that the effects of a single stressful event are far from being simply acute. Evaluating short- and long-term modifications triggered by acute stress could be a useful tool to dissect adaptive and maladaptive components underlying stress reaction.

On this basis, the aim of the present thesis was to study some early and delayed alterations caused by acute FS stress in neuronal presynaptic (synaptosomes) and astroglia peri-synaptic (gliosomes) compartments purified from prefrontal cortex (PFC) in vulnerable (VUL) and resilient (RES) rats.

We first applied the sucrose test paradigm, to identify RES or VUL rats at different time points after FS stress, thus showing that the anhedonic behaviour started 6h after FS stress and lasted at least for 48h.

FS stress was characterized by an immediate and transient rise of corticosterone levels in both VUL and RES rats respect to controls. 24h after FS stress, when no augmentation of corticosterone level was still detectable, the depolarization-evoked glutamate release was more pronounced in PFC gliosomes of VUL, but not of RES rats. No release changes were found immediately, 6h and 48h after FS stress. No modifications were detected also in basal glutamate release in PFC gliosomes of both VUL and RES rats at each time point investigated.

When focusing on PFC synaptosomes, we observed that the depolarization-evoked glutamate release was significantly increased in both VUL and RES rats respect to controls 24h after FS stress, while the basal glutamate release was increased in VUL animals only.

Studying the molecular mechanisms underlying these functional alterations, we demonstrated that the excessive depolarization-evoked glutamate release measured 24h after FS-stress in VUL PFC gliosomes was mediated by glutamate transporters operating in the reverse mode.

We also found that synapsin I expression did not change in synaptic membranes purified from PFC synaptosomes, while synapsin I phosphorylation in Ser9 was significantly increased in both RES and VUL rats, thus paralleling the increased glutamate release in PFC synaptosomes.

Moreover, mineral corticoid receptors expression was significantly increased in RES rat PFC nuclear fraction whereas that of glucocorticoid receptors was increased in VUL rats.

The present data suggest that acute FS stress induces adaptive/maladaptive stress responses at the synapse level, affecting the neuronal presynaptic counterpart and the surrounding astroglia cells with the alteration of specific molecular mechanisms that might represent new potential pharmacological targets for future therapeutic interventions.

1. INTRODUCTION

1.1. HISTORY OF THE STRESS CONCEPT

The initial contribution to stress research belongs to the North American physiologist Walter Bradfort Cannon, at the beginning of the last century. He started characterizing the adaptive responses of the digestive system after the exposure to different stimuli (strong emotions, hunger, cold, exercise, nociceptive events) and observed that the physiological functions related to the body energy reserves were suddenly boosted up or broke off during a stressful event to make a large number of resources available.

In these studies, he defined the concept of "fighting-or-flight" response to describe the wide range of mammal's behavioural reactions associated with a greater metabolic consumption as a consequence of a challenging situation (Cannon, 1915). In 1926, Cannon introduced the term "homeostasis" (Cannon, 1926), developing the concept of "milieu intérieur", coined by the father of modern experimental physiology, Claude Bernard, to describe the mechanisms which allow living organisms to keep constant the internal environment (blood pressure, blood glucose, intracellular osmolarity) in presence of external changes (Bernard, 1865). Two assumptions were primarily at the basis of homeostasis: the existence of a local regulation to promptly restore altered physiological set-points, through negative feedbacks, and the conception of organs as single functional units. Cannon expanded this concept considering psychosocial threats in the achievement of a dynamic equilibrium (Cannon, 1929, 1939).

In the 1950s, the Austrian doctor Hans Selye focused his attention on the behavioural responses to stress, observing that patients suffering from different pathologies showed a similar symptomatology (depression, mild or severe lethargy) identifiable as a single syndrome (Selye, 1936). He injected rats with different toxic substances or exposed them to extreme conditions, noticing that in all cases animals showed enlargement of the adrenal glands, atrophy of lymphoid tissue in the thymus, spleen, lymph nodes involution and gastrointestinal ulcers. He defined this pathological status the General

Adaptation Syndrome (GAS), based on a set of non-specific responses and consisting of three phases: an alarm phase which involves the activation of the sympathetic nervous system; a resistance phase characterized by the organism effort to fight against the threat; and an exhaustion phase which occurs when the organism, run out of energies, is not able to overcome the threat (Selye, 1936). Thus, for the first time a biological definition of stress was provided as "a non-specific response of the body to any demand made upon it" and the factors that trigger this reaction named "stressors" (Selye, 1950, 1976).

In the 1970s, Selye coined the expressions "distress" and "eustress" to classify the stress response according to the triggering stimulus. The first term was related to harmful, uncomfortable emotions leading to deleterious physical and psychosomatic symptoms. The second term, related to positive stress, denotes the positive individual attitude to perceive and react to events (Selye, 1974). More recently, the original definition of stress has been revised, considering environmental and psychological threats, also those predicted or perceived as of devised imminence (Schulkin et al, 1994). This broader vision led to the introduction of the concepts of allostasis, allostatic load and allostatic overload (Sterling and Eyer, 1988; McEwen and Stellar, 1993; Schulkin et al., 1994), which recognize a specificity in the response depending on the event perturbing homeostasis, the individual perception of the stressor, and the capability to face it up (Goldstein, 2001). These notions allow to better describe the intricate system having as outcome adaptation or maladaptation.

Sterling and Eyer defined allostasis as the active mechanism which implies the simultaneous variation of the biological parameters in order to realize the best match in response to chronic requests. This new stability condition does not reflect the classic homeostasis; rather, it reflects the maintenance of a renewed equilibrium (Sterling and Eyer, 1988). In allostasis, the physiological set-point continuously varies according to the stressful situation (Koolhaas et al., 2011). In this context, the involvement of systemic mediators (metabolic hormones, sympathetic/parasympathetic activity, cortisol, pro- and anti-inflammatory cytokines) is required, to obtain a network acting in a non-linear way and in which each mediator influences the others (McEwen, 2006). When resources to face further challenges exhaust, no chance to reach greater levels of reactivity occurs. This phenomenon

was described as the onset of a different set point to cope with a constant alteration of the control systems (Sterling and Eyer, 1981). An excessive use of allostatic responses due to stress accumulation or impaired mediator production translates into mounting changes which match the definitions of allostatic load and overload (McEwen, 1998).

In 1993, McEwen and Stellar defined the allostatic load as the price the organism has to pay when it incurs in forced adaptation in the presence of unpleasant psychological or physical circumstances (McEwen and Stellar, 1993). In this context, different experiences, from common life events to greater challenge or dangerous behaviours to health, such as reduced sleep, disturbance of the circadian rhythm, alcohol consumption, unhealthy diet, can be included (McEwen and Wingfield, 2010). Allostatic load can derive from different situations, such as continuous solicitations originating from simultaneous stressors, deficits in adaptation, protracted responses due to delayed switching off of the signal and ineffective reactions resulting in compensatory hyperactivity (McEwen, 1998). When individual resources are continuously stressed and unable to face environmental changes, the extreme condition of allostatic overload occurs (McEwen, 2003; Fava et al., 2019).

Most recent authors criticized the introduction of the term allostasis asserting that it constitutes only a semantic variation of homeostasis, as in both concepts the living apparatus under study remains the same (Dallman, 2003, 2005). According to this view, allostasis would only provide a minimal contribution to stress comprehension; rather, it would introduce confounding elements in the stress neurobiology field (Davies, 2016). Even though the definition of stress has changed over the centuries, the awareness that it constitutes an integral part of human existence has remained constant (Monroe and Slavich, 2016).

1.2. STRESSORS

Stressors can be defined as endogenous or exogenous challenges perceived as unpleasant from which a stress response originates when the individual regulatory capacity is no longer able to cope with the demand. Their consequences are variable and heterogeneous, depending on the properties of the stressor itself (severity, chronicity, predictability), the coping ability, the individual genetic background and/or previous life experiences (Anisman and Merali, 2000; Paykel, 2001; Kendler et al., 1992, 2001; Roy, 1985; Charney et al., 2004; Feder et al., 2009; Franklin et al., 2012; Russo et al., 2012).

The stressor effects on behavioural and biological parameters are dependent on controllability, predictability, ambiguity and uncertainty. Uncontrollable stressors induce brain region-specific neurochemical modifications comparable to those found in depression: decrease in norepinephrine (NE), dopamine (DA) and serotonin (5-HT) release in specific hypothalamic nuclei (the paraventricular nucleus, PVN) and various meso-limbic sites (central amygdala, CeA, and medial prefrontal cortex, mPFC) (Anisman et al., 1991; Deutch et al., 1993; Stanford, 1995; Heinsbroek et al., 1991; Kalivas and Duffy, 1995). When control can be exercised on stressors, stress pressure on the biological systems is less pronounced. However, the distinction between the effects of controllable and uncontrollable stressors is not always clear, especially when quick neurochemicals responses are triggered, for instance by corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) release. Unpredictability is characterized by the absence of an anticipatory response, which could concur to pathology development (Osuna, 1985). Ambiguity and uncertainty relate to situations where it is not clear whether the expected event will effectively occur (Anisman and Matheson, 2005). Basing on the perception of a stressful stimulus, stressors can be real or predicted. Real stressors imply a concrete perturbation of the homeostasis that is detected through somatic, visceral, or circumventricular sensory pathways. They produce consequences on cardiovascular tone, respiratory distress, visceral or somatic pain and on the levels of mediators involved in infection/inflammation. In predicted stressors, anticipatory responses due to conditioning (memory of past events) or species-specific dispositions (perception of predators, identification of risks linked to heights and open

spaces) are included (Anisman and Matheson, 2005). The contraposition between reactive and anticipatory outcomes is strictly related to experience, so that the environment connected to a stressor can be itself affected, translating in an anticipatory response of the predicted stimuli that will be subsequently encountered (McEwen, 1998).

One of the most important factors that contributes to determine the pathology occurrence is the ability to cope with stressors (Stowell et al., 2001; Lazarus and Folkman, 1984). The variety of possible responses induced by stressors and the dynamicity of coping strategies interfere with the ability to analyze the relation between stressful events, their appraisal and processing mechanisms (Tennen et al., 2000). Evaluations and coping strategies differ from one individual to another, depending on the situation. It follows that the specific schemes adopted are the result of a subjective construal of the stressor (Carver et al., 1989; Folkman and Lazarus, 1985). Problem-focused strategies prevail when the stressor is recognized as controllable, emotion-focused strategies (emotional expression, emotional containment, blame, avoidance, denial, and passivity) are predominant when the stressful event is perceived as something to be endured (Folkman and Lazarus, 1985; Billings and Moos, 1981).

As to duration, stressful experiences may be acute (single, intermittent and time-limited exposure to a traumatic event) or chronic (continuous, perhaps with a low intensity but for a long time). During their life, individuals most frequently endure chronic stressors which, despite their persistence over time, are characterized by inconsistence, unpredictability, ambiguity, changeability, making it difficult to set up appropriate coping strategies or restricting personal capability to plan preparatory steps. The behavioural disturbances associated to acute stressors may be not present in response to these types of insults, (Anisman et al., 1991; Anisman and Zacharko, 1982; Haleem and Parveen, 1994; Nankova et al., 1993).

Causative, stressful events can be classified into three main categories: stressors of “processive” nature, stressors of “systemic” nature and social stressors (McCarty, 1989; Pacak et al., 1998; Van De Kar and Blair, 1999).

The first group includes those that concern the evaluation of a situation or stimulus, involving higher order cortical processing and can be distinguished in neurogenic (physical) and psychogenic (psychological). Physical stressors generate actual disturbances of the physiological status, overwhelm the organism and comprehend cold, heat, intense radiation, noise, vibration, blood loss, infections, pain. Psychological stressors result from acquired responses to adverse conditions previously experienced and comprehend aversive environmental stimuli, predator-related cues, failure to satisfy internal drives. They deeply impact on emotional processes, leading to behavioural changes such as anxiety, fear or frustration (Dayas et al., 2001). They are perceived threats intrinsically linked to the critical factor of anticipation, which characterizes the conditions potentially leading to a hazard and causing homeostatic challenges (De Kloet et al., 2005a; Koolhaas, 2011). Psychological and physical stressors recruit different neuronal circuits (Ulrich-Lai and Herman, 2009) and cellular activities, inducing distinguished marks in the brain. Neurogenic stimuli in the limbic system affect the amygdala (AG), whereas psychogenic ones preferentially affect the hippocampus (HC) (Pruessner et al., 2008).

Systemic stressors are internal threats and involve physical alterations, occurring, for example, after a severe car accident. These stressful events induce a perturbation of the cardiovascular or metabolic homeostasis and comprehend exercise, orthostasis, upright tilt, heat exposure, hypoglycemia, hypovolemia and hemorrhage (De Kloet et al., 2005a; Koolhaas, 2011).

Social stressors can be defined as situations threatening interactions among individuals, personal esteem, sense of belonging to a group or to a larger social context. Their origin resides in difficult social interactions such as conflictual or tumultuous marital or family relationship, mourning, separation/divorce, detrimental and unsupportive dealings (McQuaid et al., 2014; Slavich and Irwin, 2014; Kiecolt-Glaser et al., 2010); in the context of performances evaluation where others could be judgmental or critical and in situations in which the individual feels rejected, ostracized or ignored, as in the case of unemployment, loss of a friendship, social rejection, social alienation (Dickerson and Kemeny, 2004).

1.3. NEUROANATOMY OF THE STRESS RESPONSE

After a stressful event, the organism operates at different levels to face the stressor. Two main changes can be distinguished from the point of view of the allocation of resources: modifications in pattern/amount involved in the release of energy and alterations in its distribution (Sharma, 2018). The stress response is first triggered by the perception of a stressor in the hypothalamus. When the hypothalamus meets a threat, it gives rise to the physiological stress response that can be distinguished into two different chronological processes: a very quick and a delayed phase (Lucassen et al., 2014).

The first phase concerns the "alarm reaction" or the "fight-or flight" response, involving the fast activation of the sympathetic and parasympathetic peripheral nervous systems. The preganglionic sympathetic neurons, whose somas are in the intermediolateral cell column of the spinal cord, project their axons to the postganglionic sympathetic neurons which in turn innervate the effector organs and chromaffin cells of the adrenal medulla; thus, provoking prompt alterations of the physiological condition (Charmandari et al., 2005). The resulting response is triggered within seconds, and it is quickly extinguished by parasympathetic stimulation (Ulrich-Lai and Herman, 2009). The preganglionic sympathetic neurons are rapidly activated by stress, and they act at the level of the adrenal medulla inducing an immediate release of the neurohormone adrenaline. Although adrenaline is unable to penetrate the blood–brain barrier, it can interact with the central stress circuitry by activating β-adrenergic receptors at the vagal nerve inducing the release of noradrenaline from the nucleus tractus solitarius and from the locus coeruleus (Kvetnansky et al., 2009; Wong et al., 2012). Both catecholamines produce several changes at the systemic level, such as a quick elevation of basal metabolic rate, blood pressure (due to increase of heart rate and myocardial contraction), respiration (due to bronchus dilatation), blood flow increment in heart and skeletal muscles, decrement in digestive activities (Sharma, 2018).

Later, the hypothalamic–pituitary–adrenal (HPA) axis intervenes. It represents a neuroendocrine circuit that presides the coordination of behavioural and hormonal responses to stressful events combining stimuli of cognitive, autonomic and emotional nature, mainly through the limbic and hypothalamic brain areas (de Kloet et al., 2005a; Lucassen et al., 2014). The regulation of this system

is mediated by the medial PVN, the anterior pituitary and the adrenal cortex. CRH and arginine vasopressin (AVP) are secreted by the PVN into the portal circulation of the median eminence. Consequently, the anterior pituitary, under the action of these two releasing hormones, is induced by CRH and APV to produce ACTH which, once released into the systemic circulation, stimulates adrenal glands. ACTH acts at the cortical region of the gland, triggering synthesis and release of peripheral glucocorticoid (GC), mainly corticosterone (CORT) in rodents and cortisol in humans (Ulrich-Lai and Herman, 2009; Ebner and Singewald, 2017; Spencer et al., 2018). The latter shows a circadian rhythm that follows the course of ACTH secretion: the zenith being in the morning around 7 a.m., a pre-meal secretory peak, nadir on the evening around 8 p.m. (Chung et al., 2011; Oster et al., 2006). Cortisol, to cope with the harmful effects of the stressor, mobilizes energy by conversion of glycogen into glucose and by demolition of fats into fatty acids and glycerol. Other effects include increased urea production, appetite suppression, abolition of immune system, exacerbation of gastric irritation, associated depression and loss of control (Sharma, 2018).

Glucocorticoid functions generally explicate in a slowly way through the genomic pathway acting as transcriptional regulators of glucocorticoid responsive genes (GREs), but a non-genomic path mediated by putative membrane-bound receptors has also been described (Joëls et al., 2012). HPA axis regulation involves negative feedback mechanisms and is finely controlled by cortisol binding to brain corticosteroid receptors with high affinity to mineral corticoid (MR_s) and lower affinity to glucocorticoid receptors (GR_s; de Kloet, 2005a). Both receptors concur to a circuit responsible for emotion and cognition that includes HC, AG, hypothalamus, prefrontal/frontal cortex (PFC/FC) and brainstem. MR_s and GR_s are responsible for the modulation of distinct molecular mechanisms implicated in the behavioural response to stress (Joëls and Baram, 2009). MR_s are constitutively activated in the presence of low concentrations of cortisol, present during physiological situations (Joëls, 2006; Zoladz and Diamond, 2008). These receptors allow neuronal stability, integrity of the response to a stressful event and seem to be involved also in the appraisal of a novel condition, in memory, in the modulation of behavioural flexibility, in selective attention and in anxiety-like mood (Oitzl et al., 1994; Otte, 2007; Brinks, 2007). When glucocorticoid secretion increases at the circadian peak or in presence of a stressful situation, corticosteroids also activate GR_s (de Kloet et al., 1998;

Reul and de Kloet, 1985). Thanks to the suppression of the HPA axis via a negative feedback loop and to the modulation of the MR_s activity, they support the final stage of the response, consisting in the containment of stress (Joëls et al., 2012).

1.4. DIFFERENT OUTCOMES OF THE STRESS RESPONSE: VULNERABILITY AND RESILIENCE

After having defined the physiological dynamics of the stress response, it is worth considering that not all individuals show the same reaction following exposure to a stressful condition.

Although it is well known that a continuously solicited or misadjusted system predisposes to a greater risk of developing stress-related disorders, about 80% of people exposed to traumatic situations is able to maintain a normal physical and psychological behaviour (Gold, 2015; McEwen, 2017; Han and Nestler, 2017). This phenomenon is called resilience and it has been defined as the ability to preserve a state of regular equilibrium in face of extremely unfavourable circumstances (Bonanno, 2004). The synergy of various factors, including individual neurobiological and psychological profile, personal trauma history, and peritraumatic situations, such as availability or not of social support, takes part in the determination of the response (Charney, 2004; Southwick et al., 2005; Yehuda, 2004a). Among the psychological determinants, positive emotions, optimism, active coping style, cognitive flexibility, moral compass and social support play an important role in stemming the effects of a stressful condition (Southwick et al., 2005). Early life experiences support the establishment of healthy brain structure affecting cognitive elasticity that enables to adapt successfully to the constant daily changes (Juster et al., 2010). Resilient individuals are also characterized by a peculiar reconsolidation and removal of traumatic memories, which makes them capable of attenuate learned fear through extinction (Hofmann et al., 2006; Ressler et al., 2004). Neurobiological resilience research first polarized on neural circuits linked to fear, reward, learning, social connection, and emotion regulation studying the involvement of particular brain areas such as AG, HC, insula, anterior cingulate (AC)/PFC, and the nucleus accumbens (NAc). In addition, the role of several neurochemicals, namely dopamine, norepinephrine, epinephrine, cortisol, serotonin, brain-derived neurotrophic factor, endocannabinoids, glutamate, and neuropeptide Y was analyzed to evaluate their specific contribution. More recently, a main neural mechanism concurring to resilience during extremely stressful conditions has been identified in the activation of the left PFC, which is able to transmit inhibitory signals to the AG with a resultant reduction in anxiety and fear,

as well as amelioration of the abilities to plan and act more successfully (Davidson and McEwen, 2012; Russo et al., 2012).

Differently from resilience, vulnerability has not been uniquely defined. In general, vulnerability can be described as a failure to cope with stressors because of an excessive individual sensitivity that leads to the development of inappropriate or ineffective defence mechanisms. Susceptibility to stress is connected to passive coping, characterized by denial, avoidance of conflicts, repression of emotions and behavioural disengagement (Sherrer, 2011). The vulnerable phenotype, when exposed to major stressors, shows an excessive attentional focus to the unfavourable aspects of the events which prevails and contributes to the formation of strong adverse memories, due to an amplified perception of the challenging event (Sandy and Richter-Levin, 2009). This attitude leads to maladaptive responses, thus not allowing the establishment of the resilience or providing only a short-term type one (Sherrer, 2011).

Although the mechanisms underlying vulnerability and resilience are known to involve genetic and nongenetic factors that act in complex and sequential ways, the biological basis supporting the varied range of individual stress responses and of adopted coping strategies have not yet been fully elucidated (Franklin, 2012). Nowadays the brain ability to respond to acute and chronic stress through direct modifications at the level of its architecture, molecular profile, neurochemistry is well-established (McEwen, 2017). A healthy brain is resilient and shows a marked attitude to adaptation, as it involves neural circuits whose activation provides the foundations for good self-esteem and the control for successful self-regulation, matching with specific changes in gene expression (Gray et al., 2014). Conversely, the vulnerable brain demonstrates a reduced inclination to adapt to new challenging events, no longer resulting in plastic adaptative changes or acquiring maladaptive circuitries of plasticity. The outcome of the stress responses that can lead to adaptive or maladaptive processes is based on the peculiar neurological ability of each individual, which develops according to experiences acquired during the course of his existence. Moreover, factors related to the genetic background, that can contribute in determining brain architecture, are in turn regulated by epigenetic

inputs whose consequences may lead to the promotion or failure of gene expression responses in stressful conditions (McEwen, 2017).

1.4.1 GENETIC FACTORS INVOLVEMENT IN THE STRESS RESPONSE

Meta-analyses and genetic studies have revealed that the genetic factors involved in stress-related psychopathologies concentrate in families and are to some extent heritable (Kendler et al, 2006; Gatt et al., 2015). The characteristics that contribute to delineate the genetic architecture of a phenotype consider the whole set of underlying genetic risk factors, comprehending their number, allele frequencies, and effect sizes of the concurring variants. The variations in the DNA responsible for the allelic spectrum, from which the complex phenotypic outcome arises, belong mainly to three categories: common single-nucleotide variants (SNV_s), less frequent single-nucleotide variants, and structural variants consisting of copy number variants (CNVs), insertion/deletions, and balanced translocations. The variations just described are not only hereditary, as a component of rare de novo pathogenic mutations that occurs at the gametes level is also present (Smoller, 2016). Regarding mutations linked to somatic cells, recently attention has been focused on the modifications present in subpopulations of developing or mature cells, including neurons, which have been identified as predisposing factors for vulnerability (Insel, 2014). In the context of the genetics of the stress response, genes that take part in monoaminergic neurotransmission and encode the components of the HPA axis have been largely investigated. The most extensively studied polymorphism is the functional biallelic 5-HTTLPR (serotonin (5-HT) transporter gene-linked polymorphic region) involving the promoter region of the serotonin transporter gene (SLC6A4) whose implications have been demonstrated in several psychiatric disorders (Gressier et al, 2013).

Focusing on the neuroendocrine function, a pivotal element is represented by the ADCYAP1R1 gene, encoding for the receptor of the pituitary adenylate cyclase-activating polypeptide (PAC-R1), a hypothalamic neuropeptide with regulatory effects on a wide domain of neurohormones, including stimulation of CRH secretion. Its deletion in mice correlates with a reduction in anxiety behaviour

and fear conditioning (Vaudry et al, 2009). FKBP5, FK506 binding protein 5 gene, is an important modulator of the stress responses that affects not only glucocorticoid receptor activity after stressors exposure, but also a large number of other cellular processes in both brain and periphery. It has been demonstrated that variations in this gene, derived from transcriptional and epigenetic effects on HPA axis, significantly correlate with stress-related phenotypes and disorders (Binder et al, 2004, 2008; Fani et al, 2013; Klengel et al, 2013; Mehta et al, 2011). The main functional polymorphism of BDNF gene implies a valine to methionine substitution at codon 66 (Val66Met, Martinowich et al., 2007; Willis-Owen, 2005; Schumacher, 2005), the presence of which has been linked to alterations of intracellular trafficking activity-dependent BDNF secretion, leading to a series of neuropsychiatric phenotypes, such as mood and anxiety disorders, as well as to variations in brain structure (Notaras, 2015). Nowadays, there are still few risk loci that have been correlated to an increased probability of developing stress-related disorders (Smoller, 2016; Weger and Sandi, 2018). Since this class of diseases displays a complex and polygenic nature, it is highly probable that each of the individual variants identified can be able to contribute only negligibly to the global manifestation of the vulnerable phenotype (Weger and Sandi, 2018). The study of multiple stress-related disorders in the context of family ties or in twins has shown the presence of coaggregation and co-heritability characteristics (Smoller, 2013). Moreover, the familial genomic investigations, confirmed by neuroimaging analyses, have highlighted interconnections between stress-induced diseases and other psychiatric phenotypes, as well as with subsyndromal symptoms and specific personality traits (Smoller, 2016). Although genetics has been identified as the main element responsible for the variability in the individual stress response, a large amount of clinical and preclinical research suggests that the contribution of the interaction between genetic and environmental factors is a more likely source in defining the vulnerable phenotype (Franklin et al., 2012; Klengel and Binder, 2015; Dudley et al., 2011).

1.4.2 EPIGENETIC MECHANISMS IN BEHAVIORAL RESPONSES TO STRESS

Individual phenotypes in response to stress do not arise only from the engagement of specific neural mechanisms, based on the modulation of HPA activity and neurotransmission and signalling, but also imply the involvement of processes at the chromatin level. This latter contribution is responsible of controlling the expression of genes that take an active part in stress regulation through the interaction of genetic and epigenetic factors (Franklin et al., 2012).

The term epigenetics denotes all those mechanisms capable to determine mitotically or meiotically heritable modifications in gene expression, without modifying the DNA sequence itself. These changes include DNA methylation/demethylation on cytosine residues, covalent post-translational modifications (methylation, acetylation, phosphorylation, ubiquitylation, sumoylation) of histones that suppress or trigger chromatin unfolding, non-coding RNAs insertion, transposons, retrotransposons and RNA editing (Franklin et al., 2012; Allfrey, 1970; Mehler, 2008; Griffiths and Hunter, 2014; Mehler and Mattick, 2007).

More in detail, DNA methylation and histone acetylation are the two mechanisms that have been respectively related to stress susceptibility and resilience with particular reference to the onset of depressive symptoms in both animals and humans. The first mechanism is capable of inducing depressive features and this assessment derives from the studies in animal models which have received reduced maternal care in their early life. They showed a greater degree of DNA methylation, resulting in an inhibition of the expression of corticosteroids receptors, and demonstrated anxious-depressive behaviours (Tsankova et al., 2006). The second mechanism is associated to the activation of transcription and with an antidepressant action. At this regard, it has been reported that an increased acetylation of histones containing the gene for the BDNF receptor results in a lower incidence of depressive manifestations (Tsankova et al., 2006).

Nevertheless, it has been observed that histone acetylation in the brain can significantly vary in response to chronic stress giving rise to distinct effects according to the different cerebral areas. In the NAc, a decrease in the expression of HDAC2 and HDAC5 (two isoforms of the enzymes that

catalyze the removal of acetyl functional groups from the lysine residues of both histone and nonhistone proteins) has been correlated with a depressive syndrome (Renthal et al., 2007; Covington et al., 2009). In the HC of stressed animals, the total H3K14 acetylation, an epigenetic modification to the DNA packaging protein Histone H3, presents a transitory enhancement after social defeat stress, whereas HDAC5 is remarkably reduced after unpredictable stress (Covington et al., 2011; Sterrenburg et al., 2011). In rats PFC, reiterated stress induces an increase in HDAC2 expression, producing the epigenetic variation of Nedd4, an E3 ubiquitin ligase involved in α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) degradation, and causing a damage in AMPAR expression and cognitive function (Wei et al., 2016).

An important element related to the adapting ability in response to stress, consequently leading to resilience, is the interaction between genes involved in the function of the HPA axis and the exposure to chronic social defeat stress. In fact, CRH expression is high in the hypothalamus of animals that, after the stressful event, develop social avoidance and the CRH gene is hypermethylated in the subset which does not present stress-induced social avoidance, (Elliott et al., 2010; Moncek et al., 2004). Further, the possible existence of the interplay between genetic variants and environmental factors in the onset of stress susceptibility, such as the mutated number tandem repetitions of the Monoamine oxidase A gene (MAOA) (Cicchetti et al, 2007), the T102C polymorphism of the Serotonin receptor 2A gene (HTR2A) (Jokela et al., 2007), the single nucleotide polymorphisms of the Corticotropin releasing hormone receptor 1 gene (CRHR1) (Bradley et al., 2008), or the 22/23EK and 9beta polymorphisms of the Glucocorticoid Receptor gene (NR3C1) (Bet et al., 2009), has been highlighted.

In addition, the use of pharmaceutical interventions, the actuation of health-improving strategies, like cognitive-behavioural therapy, physical activity, and social support promotion has been reported to revert epigenetic modifications in determined time windows (McEwen et al., 2014).

1.4.3 MALADAPTIVE RESPONSES TO STRESS

In the past decade, it has become acknowledged that a prolonged or disproportionated stress response is able to induce maladaptive changes, which in turn are responsible for the onset of mental and physical disorders. In the central nervous system (CNS), these alterations concern the structure and function of neuron synapses, affecting excitatory/inhibitory circuitry and having a primary role in the pathophysiology of memory deficits, impaired cognition, mood and anxiety disorders (MADI), especially major depression (MD, Popoli et al., 2012; Tokita et al., 2012). Other stress-related disorders exert their effects in the peripheral nervous system and in many different organs, leading to cardiovascular (cardiac arrhythmias, angina, congestive, heart failure, hypertension and/or cardiac hypertrophy), immunological, gastrointestinal and metabolic abnormalities (Antunes-Rodrigues et al., 2005; Lundberg, 2005; McEwen, 2007; Dünser and Hasibeder, 2009; Zhang and Anderson, 2014; Carter and Goldstein, 2015; Tank and Lee Wong, 2015; Breen et al., 2016).

Increasing evidence prove the existence of common pathogenic mechanisms among the disabling comorbidities induced by stress, such as depression, anxiety disorders, post-traumatic stress disease (PTSD) and epilepsy. Although the relationship between the underlying processes is still unclear, the most accredited hypothesis assumes that the presence of structural and functional modifications induced by a given disorder may trigger the others (Gold et al., 1988; de Kloet et al., 2005a, b; Kanner, 2012). On the other hand, the role of the stress hormones in the predisposition to different psychopathologies is well established and is considered the root cause of emotional arousal and psychic disorganization rather than the reason of specific disorder per se (Sachar et al., 1970). A proof of this assumption is represented by the Cushing's disease that is characterized by depressive symptoms and whose remission can take place through surgical correction of the hypercortisolemia (McEwen, 2003). The chronic increase of cortisol is a feature shared by both MD and Cushing's disease, causing in turn a gradual loss of minerals from bone and abdominal obesity. Structural magnetic resonance imaging highlighted a continuous decrease in HC volume, also detected in a variety of other anxiety-related disorders, highlighting this event as a common index of chronic imbalance of the adaptive system activity, suggesting the involvement not only of the HPA axis but

also of neurotransmitters such as glutamate (Glu, Sheline, 2003; Sheline et al., 1999; McEwen, 2005). Furthermore, the HPA axis stress-operated dysregulation can constitute the trigger that determines the establishment of immunological responses, whose consequences are retained as risk factors for Alzheimer's disease (Jeong et al., 2006; Caruso et al., 2018; Hoijemakers et al., 2016).

Depression and anxiety are often two concomitant pathological aspects presenting, as distinctive characteristics, impairment of health, cognitive, and emotional functions (Kroenke et al., 2007). The connection between these two disorders and the pivotal role of stress in their onset are further confirmed by the evidence that chronic treatment with CORT produces depressive-like symptoms but also causes AG hypertrophy and an enhancement of anxiogenic behaviour (Mitra and Sapolsky, 2008). Hyperactivity of the locus coeruleus – norepinephrine (LC-NE) system, the main target of CRH, is involved in the development of stress-related psychiatric disorders (PTSD, MD), typical manifestations of which includes hyperarousal, loss of concentration, restiveness and compromised focused attention (Southwick et al., 1999; Wong et al., 2000).

PTSD patients have shown rising rates of anxiety and depression, although low levels of cortisol have been found (Nemeroff et al., 2006; Daskalakis et al., 2013; Zoladz and Diamond, 2016; Zorn et al., 2017). However, this disorder allowed to identify dysregulations of the stress response in other compartments of the HPA axis, regarding its enhanced negative feedback, accentuated GR sensitivity, increased levels of CRH in the cerebrospinal fluid, and decreased ACTH release after treatment with CRH and CCK4 (a peptide fragment with potent anxiogenic properties) (Grossman et al., 2003; Duval et al., 2004; Yehuda et al., 2004b; Rohleder and Karl, 2006; Bremner et al., 1997; Baker et al., 1999; Smith et al., 1989; Kellner et al., 2000). In PTSD, these neuroendocrine modifications are induced by exposure to acute stressors, such as urban violence, sexual abuse, combat in war and disasters, which are also responsible for the manifestation of physiological and behavioural alterations comprehending nightmares, hypervigilance, flashbacks of the trauma and sleep disturbances in affected individuals (Zoladz and Diamond, 2013; Yehuda et al., 2015).

As the prevalence of stress-related disorders is continuously increasing, the need for the identification of the mechanisms linked to stress and for the development of new pharmacological therapeutic approaches becomes crucial and urgent.

1.4.4 THE PFC AS A TARGET OF MALADAPTIVE RESPONSES

1.4.4.1 THE PFC: FUNCTIONS AND STRUCTURE

Although stress is known to induce marked behavioural changes, as depressive-like symptoms, hyper anxious states and learning and memory deficits predominantly involving the HC, other stress-related alterations, such as deficits in executive function, are not attributable to this brain area (Mizoguchi et al., 2000; McEwen, 2004). Another key cerebral region is represented by the PFC, the function of which that can be influenced by stress includes the working memory, articulated in temporary retention and handling of information to manage subsequent behaviours (Goldman-Rakic, 1995). The PFC also plays a predominant role in the elaboration of emotional stimuli and in the complex cognitive tasks, consisting of temporal arrangement of behaviour, decision-making, rule learning and behavioural elasticity (Davidson, 2002; Clark et al., 2004). PFC includes several areas of the frontal lobe, that are highly developed in humans compared to other species, composed of numerous sub-areas anatomically distinct (Uylings and Groenewegen et al., 2003; Chudasama and Robbins, 2006). The PFC can be divided into two main areas: the medial dorsolateral (mPFC) and the orbitofrontal (OFC) area (Fuster, 2001). The mPFC, in turn, includes the frontal 2 (Fr2) area, the dorsal and ventral areas of the anterior cingulate (ACd and ACv), the prelimibic (PL), infralimbic (IL) and mid-orbital (MO) regions. The OFC area is divided into the insular dorsal area (AId), the anterior ventral insular area (AIv), the orbital lateral (LO), and the ventral orbital (VO) areas (Zilles and Wree, 1995; Dalley et al., 2004).

The PFC shows several connections with the basal ganglia and the thalamus, especially with the middle dorsal thalamic nucleus through which the subcortical limbic information, including those originating from the thalamus, are transmitted to the ventral areas of the mPFC. This specific area receives several afferents from the limbic regions, such as the AG and the ventral subiculum, providing direct outputs to the hypothalamus and to numerous areas of the brain involved in emotions

regulation and in the physiological stress-response (Bandler et al., 2000). In particular, the PFC is related to the HC by projections having their origin in the subiculum and ventral CA1 sub-regions and projecting in the pyramidal cells and interneurons of the mPFC (Jay and Witter, 1995; Tierney et al., 2004). Learning and memory processes and the stress response are involved in a synergistic control that derives from the synaptic activity modulation through the interplay between these two brain areas (Wal and Messier, 2001; de Kloet et al., 2005a). As to the first aspect, it has been observed that training in associative learning task can trigger the increase of synaptic transmission in the hippocampal-PFC pathway (Doyère et al., 1993). Focusing, instead, on the regulation of the HPA axis function, the implication of both the cerebral regions in the inhibition of CRH in the paraventricular hypothalamic nuclei is of relevant importance (Sullivan and Gratton, 2002; de Kloet et al., 2005a). Also, the ventral and dorsal areas of the PFC, thanks to the numerous connections with the previously mentioned brain regions, exercise an important control over the autonomic nervous system. Electrical stimulations in the dorsal areas (prelimbics/AC) activate the parasympathetic neurons, whereas excitation of the ventral regions typically elicits sympathetic responses (Powell et al., 1994). It is of considerable interest that patients with damages to the PFC ventromedial regions often fail to exhibit automatic responses to emotional stimuli and show severe deficits in social, decision-making and risk assessment tasks (Damasio et al., 1990).

1.4.4.2 ROLE OF THE PFC IN THE STRESS RESPONSE

In the 80 of the last century it was demonstrated for the first time that the stimulation of the PFC leads to an increase of corticosteroid levels in the blood (Frankel and Jenkins, 1975; Feldman and Conforti, 1985). In the subsequent years, the complex contribution of PFC to the regulation of the HPA axis has been elucidated (Sullivan and Gratton, 2002; Wang et al. 2005; Pruessner et al., 2008). The PFC activates the HPA axis in a stimulus-specific way: only the mPFC ventral and dorsal portions are involved in the regulation of physiological and behavioural responses to stress (Diorio et al., 1993; Spencer et al., 2004). The dorsolateral regions, instead, are related to the decrease of behavioural reactivity, which is implemented through an increase of the parasympathetic system activity and a reduction of the HPA axis activation (Sullivan, 2004; Radley et al., 2006). As regards the ventromedial areas, in particular the infralimbic (IL) region, they induce the emotional responses

triggering the activation of the sympathetic system and the HPA functioning (Sullivan and Gratton, 1999; Radley et al., 2006).

In detail, the IL and PL regions of the mPFC play a key role in the resilience of fear memories to extinction, a mechanism involved in the maintenance of fear and anxiety symptoms which characterize PTSD (Giustino and Maren, 2015). Studies aimed at comprehending the neural processes supporting learned fear and its extinction has shown a steady increase, because of the prevalence of stress and trauma-related diseases (Quirk and Mueller, 2008; Milad and Quirk, 2012; Maren et al., 2013). Although it is well known that AG and HC are central in conditioned fear and extinction, a dichotomic function has been suggested regarding the mPFC, where PL exert the expression of fear, as opposed to IL, which is associated with an alleged suppression of the circuit (Quirk and Mueller, 2008; Sotres-Bayon and Quirk, 2010; Milad and Quirk, 2012; Maren et al., 2013). The hypothesis according to which the neural processes of extinction learning is maintained across species is supported by the fact that despite these two subdivisions of mPFC have been investigated in rodents, an almost identical partition of assignment has been propounded in humans (Phelps et al., 2004; Schiller et al., 2008; Sehlmeyer et al., 2009; Milad and Quirk, 2012; Vervliet et al., 2013).

More recent studies focused on anatomical and electrophysiological features have revealed that PL and IL show similar projection to the AG with a covariation of their neuronal activity when the phenomena related to fear conditioning and extinction take place (Gutman et al., 2012; Pinard et al., 2012; Cho et al., 2013; Hübner et al., 2014; Morrow et al., 1999; Baeg et al., 2001; Frankland et al., 2004; Herry and Mons, 2004; Kim et al., 2010; Holmes et al., 2012; Fitzgerald et al., 2014, 2015a; Halladay and Blair, 2015). In light of this new evidence, situations in which IL and PL activity act according to a functional dichotomous pathway have been confirmed, but with the detection of an opposite operational mode compared to the that predicted by the canonical model (Chang et al., 2010). However, novel insights to ameliorate actual therapeutic outcomes and to restrain unsuitable fear responses are required for a knowledge of the fear circuit in rodents and humans (Giustino and Maren, 2015).

1.4.4.3 STRESS EFFECTS ON PFC: IMPAIRED COGNITIVE FUNCTIONING

Another relevant brain prerogative is cognition, an articulated process that encompass stimuli perception, reception, and interpretation; this latter including learning, attention, judgment and decision making (Sandi, 2013). Decision making requires the main involvement of the prefrontal lobes and is implied in several executive functions, such as planning, organization, maintenance of the attention focus, execution of complex tasks aimed at achieving a goal, and flexibility of response in relation to different environmental contingencies. An efficient decision-making, therefore, requires the correct functioning of more cognitive processes. Studies conducted to investigate the relationships between stress and cognitive functioning have, at first, highlighted that stressful situation can have negative effects on cognitive processes, with important detrimental outcomes on individuals behaviour (Al'Absi et al., 2002; Ramlow et al., 2005; Preston et al., 2007; Starcke et al., 2008). These consequences exhibit different degrees of severity, according to stress intensity, duration, origin, and magnitude (Sandi, 2013).

It is now well established that stressful conditions exert their effects on cognition both in the short and long-term (McEwen and Sapolsky, 1995). In general, studies have shown that a reduction of glucocorticosteroids secretion induces neuroprotective functions, even if other studies have demonstrated that the effects of adrenal steroids might be variegated, since hydrocortisone injection in correspondence of its maximum plasma concentration causes a decrease in reaction time and an improvement in cognition (Lupien et al., 2002). The studies in the literature have focused mainly on short-term stressor effects, given the greater ease of their managing with respect to long-lasting stressful conditions.

1.4.4.4 STRESS EFFECTS ON MEMORY

One of the most important features of the CNS is memory. Frontal and parietal lobes are involved in short term memory, whereas long term memory is attributable to wider brain regions (Wood et al., 2000). Nevertheless, the global memorization, including modification from short to long term memory, is mediated by HC, a cerebral structure rich of glucocorticoid receptors (Scoville and Milner, 1957; Asalgoo et al., 2015). The negative effects of acute stress in this area cause structural changes, such as atrophy and neurogenesis disorders (Lupien and Lepage, 2001). At this level, the consequences of prolonged exposure to stressors are characterized by a reduction in the number of dendritic branches, of the amount of neurons and by structural modifications at the synaptic terminals and a decrease in hippocampal neurogenesis, events related to plasma cortisol increase (Woolley et al., 1990; Sapolsky et al., 1990; Gould et al., 1998). Elevated levels of stress hormones cause deficits particularly in declarative memory, the conscious remembrance of experiences, which is mainly regulated by the HC compared to other types of memory (Lupien and Lepage, 2001). However, preclinical studies have demonstrated a link between HC atrophy, one of the most relevant changes induced by excessive plasma glucocorticosteroid, and the reversible decrease in spatial memory (Luine et al., 1994; Issa et al., 1990). Stress also interferes with memory formation and, in particular, with long-term potentiation (LTP), acting at both the aspects involved in this process: the emotional component of memory, located in the basolateral AG area, and its consolidation through corticosteroid intervention (Bliss and Lømo, 1973; Joëls et al., 2011). However, even in this case, alterations in memory process may occur, leading to AG inhibition, if the levels of stress hormones are not finely regulated (Joëls et al., 2011).

1.5 PRECLINICAL MODELS TO STUDY STRESS CONSEQUENCES

The last three decades have been crucial in overcoming the challenge constituted by the need to study higher brain functions while having to deal at the same time with ethical and practical limits for the investigation of the living human brain. Animal models have represented a substantial progress in the study of stress-related disorders, although limitations still remain in investigating peculiar aspects of the physiology and molecular biology of the human brain, due to the subjective nature of many symptoms, the deficiency of biomarkers, the lack of objective diagnostic tests and the intrinsic contribution of mostly unknown genetic factors (Nestler and Hyman, 2010). In general, the essential criteria to evaluate whether a particular disease model is reliable enough for the investigation of a disorder are mainly three: the construct, the face and the predictive validity. Construct validity refers to how relevant to the disease are the methods for the construction of the model; for this reason, the ideal condition refers to the reproduction in animals of the etiologic mechanisms that determine the disease in humans, thus allowing to replicate neural and behavioural features of the illness (Chadman et al., 2009). Face validity means that a model reassumes prominent anatomical, biochemical, neuropathological, or behavioural features. Predictive (or pharmacological) validity denotes the ability of a model to respond to treatments in a way predictive of those treatment effects in humans (Nestler and Hyman, 2010).

The most frequently used animal models for the study of the mechanisms underlying neuropsychiatric diseases involve the use of chronic stress protocols (Katz et al., 1981; Nestler and Hyman, 2010; McEwen et al., 2014). The exposure for several weeks to everyday immobilization, a selection of randomized stressors, and/or repetitive social stressors, such as the presence of a dominant male, has demonstrated to be able to induce in animals phenotypical alterations comparable to those detected in patients suffering from MD (Willner et al., 1992; Berton et al., 2006; Donahue et al., 2014; Naert et al., 2011; Willner, 2017). In fact, at the end of these stress protocols animals show anhedonia that represents a core symptom of this stress-related disorder, although it is not a peculiar sign of depression since is observed also in schizophrenia and stimulant withdrawal, and a promising target for research (Nestler and Hyman, 2010). Moreover, repeated stressors proved to be useful also in

mimicking functional and behavioural alterations (cognitive impairment, modifications in working memory and fear extinction), and pathophysiological changes (dysregulation of the HPA axis, modified neurotransmitter levels) closely connected to stress-related neuropsychiatric disorders (Garcia et al., 2009; Hill et al., 2012; Russo et al., 2012; Tornese et al., 2019; Liston et al., 2006; Hains et al., 2009; Eiland et al., 2012; Musazzi et al., 2015).

The main evidence of neuroarchitecture and function alterations in specific brain regions have been detected by using chronic stress protocols. Nevertheless, it has been suggested that considering only the consequences produced by exposure to repeated stressful conditions may not represent the best procedure to evaluate the mechanisms by which the physiological stress response can shift towards maladaptive pathways, greatly increasing the probability to develop the disease (Nestler and Hyman, 2010). This statement lies on the ability of chronic stress to reproduce only the endpoint of several adaptive modifications during the stress response (Musazzi et al., 2017). Moreover, it has been reported that a single acute stress does not give origin only to short-term consequences but also to sustained pathological alterations in vulnerable subjects (Hagenaars et al., 2011; Musazzi et al., 2017). In light of these issues, it has emerged the need to introduce acute stress protocols as a tool to evaluate the mechanisms underlying maladaptive responses to stress, taking into account that no single model can entirely reproduce depression or other diseases (Musazzi et al., 2017).

1.6 ASTROCYTES IN THE STRESS RESPONSE AND MOOD DISORDERS

Understanding the mechanisms deriving from genetic predisposition to synaptic, neuronal network, and behavioural alterations in stress-related disorders has recently led to a research shift towards the involvement of glial cells (Murphy-Royal C. et al., 2019). The evidence regarding neuronal cells and stress are extensively consolidated, while too little is still known about the role of glia in the stress response (Bains et al., 2015; Joëls and Baram, 2009; Lupien et al., 2009; Ulrich-Lai & Herman, 2009). Among the several functions involving glia (neuronal activity, synaptic plasticity, blood flow), the influence on behavioural features is also included.

Growing evidence attributes a key role to astrocytes in acute stress events, considering the genetic background as a main concurring component (Ongür et al., 1998). MDD exerts effects on glial cells density especially at the level of the left AG with a direct correlation between alterations in the grey matter volume and the severity of the disease (Zavorotnyy et al., 2018). Some reports highlight a decrease in astrocytes number in individuals affected by MDD not treated with antidepressants (Cobb et al., 2016) and the same phenomenon has been detected in patients with bipolar disorders (Bowley et al., 2002). These data suggest that mental disease type can determine different astrocyte density variations within a specific brain area (Bowley et al., 2002; Ongür et al., 1998). and imply that the drugs used for the treatment of mood disorders probably exert their effects thanks to the rescue of this cell type population.

Another element relates to the presence of astrocytes-specific structural changes (Miguel Hidalgo et al., 2000; Torres-Platas et al., 2011) showing that astrocyte size increases with aging of depressed patients (Miguel-Hidalgo et al., 2000) as well as process length and cell body volume (Torres-Platas et al., 2011). Astrocyte morphology is tightly connected to synaptic function, suggesting that the morphological alterations taking place in MDD brain tissue could condition neuron–glia interactions at the synapse level (Henneberger et al., 2018; Oliet et al., 2001; Ostroff et al., 2014; Pannasch et al., 2014). Studies highlight that acute stress effects on astrocytes induce significant alterations on behaviour. Astrocytes, following a single severe stressful event, release fibroblast growth factor 2

(FGF2), whose action stimulates the maturation of hippocampal neural stem/progenitor cells (NPCs) and allows retention of fear extinction memory (Kirby et al., 2013).

FGF2 is also fundamental for antidepressant rescuing of depressive behaviour (Elsayed et al., 2012). The FGF system has been described as a clinically relevant target in MDD, since its downregulation responds to SSRIs (Evans et al., 2004), and in PTSD, since FGF2 administration ameliorates the behavioural impairment triggered by a single prolonged stress protocol (Xia et al., 2013). In the hippocampal region treated with FGF2, astrocytes have shown a decrease in GFAP and structural changes, highlighting a link between astrocytic damage and behavioural deficits (Xia et al., 2013). Also, the use of a single inescapable foot shock protocol (Saur et al., 2016) induced the same GFAP expression and morphology changes, suggesting a retained astrocytes response towards acute stress.

Another important aspect regards the effects that both acute and chronic glucocorticoid in vivo treatments in rodents exert on astrocytes (Carter et al., 2013). Transcriptomic analysis allowed to identify the glucocorticoid-mediated pathways in stress diseases, that could represent a leading mechanism of astrocytes dysfunction. These results have been confirmed in MDD patients (Bernard et al., 2011; Ernst et al., 2011; Nagy et al., 2015). Nevertheless, the acute treatment with glucocorticoid was not able to integrally reproduce the synaptic phenomenon (MacDougall and Howland, 2013), leading to the speculation that synergistic effects, mediated by parallel neurochemical stress-related cascades, regulate modifications of the synaptic function (Roebuck et al., 2018).

Other studies have been conducted on purified cell cultures to evaluate the effects of antidepressant drugs on astrocytes. The in vitro and in vivo capability of SSRI and TCA to enhance the expression of gap-junction channel proteins has emerged (Jeanson et al., 2015; Mostafavi et al., 2014; Fatemi et al., 2008; Morioka et al., 2014). Moreover, antidepressants induce the release of neurotrophic factors such as VEGF (Allaman et al, 2011) and BDNF (Allaman et al., 2011; Hisaoka-Nakashima et al., 2016; Kittel-Schneider et al., 2012; Quesseveur et al., 2013) from astrocytes.

It seems clear that astrocytes alteration is a leading driver in the definition of depressive phenotypes and that the therapeutic effects of several antidepressants may derive from a direct intervention on

these cells, which are main actors in multiple aspects of stress disorders (Murphy-Royal C. et al., 2019).

1.7 ALTERATIONS INDUCED BY ACUTE STRESS ON BRAIN MORPHOLOGY

1.7.1 CONSEQUENCES AT THE SYNAPTIC LEVEL

Synaptic transmission, plasticity, and memory are three fundamental functions mainly mediated by excitatory synapses whose activity has been found to be modulated by stress and glucocorticoids in an active and dynamic way (Krugers et al., 2012; Timmermans et al., 2013).

Sprouting of excitatory synapses and enhancement of the number of docked vesicles have been detected after exposure to an acute stressor, as consequence of strengthening of excitatory neurotransmission through induction of presynaptic structural plasticity (Nava et al., 2015). These modifications especially concern pyramidal neurons of layers II-III of mPFC, giving unique sensitivity to chronic stressful events (McEwen and Morrison, 2013). Surprisingly, acute stress has proved capable to produce changes at this specific cerebral area by increasing the number of docked vesicles exclusively in perforated synapses (PSs), featured by a discontinuous postsynaptic density and possibly representing single functional connections (Treccani et al., 2014; Jones, 1993). The most accredited hypothesis at the basis of the phenomenon is based on an increased presynaptic release probability, sustained by the rise of the number of vesicles in the reserve pool able to become competent for fusion (Musazzi et al., 2010; Schikorski and Stevens, 2001; Sudhof, 2004). The intensity of the acute stressor exerts an important contribution in modulating synaptic enhancement: a severe stimulus is able to determine a strong and quick asymmetric synapses increase, an event that occurs to a lesser extent in the presence of a mild stressful condition (Treccani et al., 2014). The axo-spinous asymmetric synapses of the NPS_s, characterized by reduced synaptic efficacy, also mediates an effect similar to PS_s after acute stress. However, NPS_s enhancement correlates with the number of silent synapses, excitatory synapses non-conducting at resting membrane potentials, which mostly express N-methyl-d-aspartate receptors (NMDARs) and only a small or absent amounts of α-amino-3-hydroxy-5-methyl 4-isoxazole propionate receptors (AMPARs; Isaac et al., 1995). Due to this peculiar characteristic, they are a valid substrate for the insertion of AMPARs, a fundamental step in

the generation of new functional connections (Suvrathan et al., 2014). Ex vivo evidence have demonstrated that this phenomenon takes place 40 minutes after the exposure to an acute stressful event, probably due to modifications in dendrite proteins during the translation phase. In this context, BDNF is responsible for the translocation towards the synapse of a subgroup of dendritic mRNAs, which comprise those coding for key cytoskeletal proteins for synaptic readjustment (Leal et al., 2014). According to this view, acute stress consequences would occur also at the postsynaptic level, leading to strengthening of NMDARs and AMPARs expression in PFC and to changes of the kinetics of excitatory postsynaptic current (EPSC) (Yuen et al., 2009; Musazzi et al., 2010).

1.7.2 CONSEQUENCES ON DENDRITES AND SPINE DENSITY

Dendritic spines can be defined as small (1-10 μm) projections located on neuronal dendrites, that, once mature, represent the sites of the postsynaptic density (PSD), including a variety of relevant postsynaptic proteins, neurotransmitter, neurotrophins, and hormone receptors (Herms and Dorostkar, 2016).

Compartmentalization of neuronal signalling is highly dependent on the distinctive structure, localisation and motility of dendritic spines (Nimchinsky et al., 2002). The essential involvement of these protrusions in synaptic functioning has made them a heavily investigated element in studies on stress consequences, since their significant contribution in brain volumetric alterations due to stressful conditions has been hypothesized (Keifer et al., 2015; Kassem et al., 2013). It is becoming increasingly more evident that stress is capable to induce not only dendritic retraction but also removal of the post-synaptic sites at the level of PFC and HC, along with overall significative reduction of dendrites ability to form efficient synapses (Kaul et al., 2021). In rodent models of chronic stress, an important loss of dendritic spines in the PFC and in the CA3 region of the HC has been identified, whereas the effects are less clear, whether increase or decrease in spine density occurs, in the CA1 hippocampal area (Helmeke et al., 2009; Yang et al., 2015a; Moda-Sava et al., 2019; Goldwater et al., 2009; Conrad et al., 1999; Orlowski et al., 2012; Stewart et al., 2005; Kassem et al., 2013; Magarinos et al., 2011).The ability of acute stress to induce an increase in spine density in hippocampal CA1 of male rats within 24h from the application of the stressful event has been demonstrated (Shors et al., 2001). This observation has proved to be consistent with the results of

previous research exposing HPC slices to CORT for 1h and showing an enhancement in spine density in the same area (Komatsuzaki et al., 2012). Nevertheless, an acute restraint stress protocol has highlighted an opposite effect in CA3, leading to a fast rise in spine density (Chen et al., 2008). Other studies focused on dendritic remodelling in the prelimbic (PL) region of the PFC showed an enrichment of spine density, at least up to 1 day after exposure to the stressful event, and the onset of a substantial atrophy of apical dendrites, starting from 1 day after stress and maintained up to 14 days (Nava et al., 2017).

1.7.3 CONSEQUENCES ON NEUROGENESIS

Several studies have produced the prerequisites to believe that also the HC is implied in the regulation of mood, by proving its vulnerability to numerous hormones released as a result of stressful experiences (McEwen, 1999).

In particular, the hippocampal region most affected by the stress effects is the dentate gyrus (DG), which is responsible for the continuous production of new neurons throughout adulthood. A reduction in adult neurogenesis is usually linked to the development of a depressive-like phenotype (Wong and Herbert, 2004; Mineur et al., 2007). A decrease of hippocampal neurogenesis, induced by a protocol not involving stress application did not lead to mood disorders, indicating that decreased neurogenesis accelerates depression-like symptoms due to an impairment of stress regulation (Petrik, 2011). Other studies highlighted the role of adult neurogenesis in strengthening glucocorticoid-mediated negative feedback on the HPA axis, as a reduction in neuronal proliferation induced by genetic alterations or exposure to radiation directly correlates with a rise of stress hormone levels after stressors administration (Egeland et al., 2015). Adult neurogenesis could be also involved in a process known as pattern separation, whose compromission by stress determines the absence of experience distinction or the presence of stimuli that are no longer coded into divided representations. The result is an overlap of pieces of information without the possibility to distinguish them in memory; making events, that are normally judged as innocuous, aversive (Cameron and Gould,1994; Gould et al., 1998; Sahay et al., 2011; Aimone et al., 2011). The loss of this skill, likely due to a reduction of adult neurogenesis, leads to overgeneralization, in which past experiences are catalogued as negative memories and unfavourable recollection occurs (Kheirbek et al., 2012). Indeed, the buffering function of neurogenesis may induce alterations of stress perception by emphasizing the flexibility of contextual emotional processing, a mechanism allowing the instantaneous distinction between

adverse and harmless events (Egeland et al., 2015). It is therefore plausible that the deficiency of neurogenesis alters the correct stimulus perception. This modification can trigger a stress response in the absence of real threat, just like in individuals suffering from mood disorders.

Several researchers highlighted that both acute and chronic stress impact on adult neurogenesis, mainly acting on cell proliferation (Egeland et al., 2015). Despite acute stress is generally reported to have suppressive consequences, in numerous and more recent studies evidence have emerged of no effects or even of its ability to increase aspects of neurogenesis. In detail, an enhancement in cell proliferation in the dorsal DG and a memory improvement have been highlighted after exposure to various acute stressors, such as foot shock (FS), immobilization, and exposure to a novel environment (Kirby et al., 2013). It is also noteworthy that a rise of stress hormone levels, induced by non-aversive activities like acute exercise and sexual experience, produces, as a consequence, an enhancement of adult neurogenesis in the rodent HC, suggesting the intervention of other factors which contribute to determine the effects on hippocampal cell proliferation (Kronenberg et al., 2006; Leuner et al., 2010).

Glutamatergic neurons represent about 80% of the total neuronal cells in the neocortex and intervene forming 85% of synapses (Douglas and Martin, 2007). Morphological alterations of their structure are strongly interconnected with functional changes deriving from a sustained glutamate accumulation. Causes are identifiable in altered glutamate release, clearance, and metabolism in selected brain areas (mPFC, HC, AG) linked to cognitive-emotional behaviours and mood regulation (Musazzi L. et al., 2014a).

The higher and sustained extracellular concentrations of the excitatory amino acid neurotransmitter are also related to dendritic retraction and impairment of glial and neuronal function; whereas typical physiological mechanisms traceable to activity-dependent synaptic plasticity are represented by modifications in the size and shape of dendritic shafts and spine density (Musazzi L. et al., 2014a).

1.8 ALTERATIONS INDUCED BY ACUTE STRESS ON BRAIN FUNCTIONS

1.8.1 CONSEQUENCES ON SYNAPTIC PLASTICITY

The two most studied mechanisms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD). LTP is defined as the phenomenon resulting from the conduction of a short, high-frequency, train of electrical excitement, which is able to determine a long-lasting activity-dependent enhancement in excitatory synaptic strength. On the other side, the effect of a low-frequency stimulation leading to a long-lasting reduction in synaptic effectiveness takes the name of LTD (Bliss and Collingridge, 1993; Abel et al., 1997; Malenka and Nicoll, 1999; Martin et al., 2000; Lynch, 2004; Malenka and Bear, 2004).

Experimental data have shown that in some brain areas both phenomena take place due to the activation of AMPA and NMDA receptors and through modifications of Ca^{2+} concentrations [Ca^{2+}] at the postsynaptic level. The currently most accepted hypothesis refers to the fact that the increase of [Ca^{2+}], triggered by the high frequency activation of the NMDA receptor is capable to produce a persistent rise in neurotransmitter release evoked by low stimuli frequency. Metabotropic glutamate receptors located in postsynaptic regions can also be involved in the genesis of LTP through neurotransmitter release enhancement or inhibition and an upstream modulation in the functioning of ionotropic receptors (Riccardi et al., 2011). In recent decades, the high susceptibility of HC, the main area involved in LTP, to stress has been highlighted both in clinical and pre-clinical research (Kim and Diamond, 2002). More in detail, the intensity and frequency of the stressor (mild, acute, chronic) and the affected hippocampal area (CA1, DG, ventral or dorsal HC) cover crucial roles in defining the stress-induced modifications of LTP (Kumar, 2011). According to the traditional view of the action of stress on LTP and memory, stress effect is represented by an inverted U-shape curve in which a low-mild stress level would facilitate whereas an excessive stress would impair LTP induction (Diamond et al., 1992; Joëls, 2006).

However, this model does not fully reflect the real consequences of stress, especially after the discovery that CORT released after exposure to a stressful stimulus acts on its membrane-bound receptors (mMR and mGR) also through novel non-genomic pathways, affecting ionic conductance and altering cell excitability (Maggio and Segal, 2010; Karst et al., 2005; Karst and Joëls, 2005; de

Kloet et al., 2008; van Gemert et al., 2009). Both chronic and acute stress have demonstrated to abolish LTP at the hippocampal synapses (Foy et al., 1987, 2008b; Shors et al., 1989; Xu et al., 1997; Pavlides et al., 2002; Alfarez et al., 2003; Diamond et al., 2005; Artol et al., 2006; Kavushansky et al., 2006; Foy.; 2011). In the presence of the same stressor, effects on LTP can vary, and sometimes be opposite, depending on the affected brain area: chronic psychological stress impairs the induction of LTP in hippocampal area CA1, whereas it does not cause alterations in DG (Gerges et al., 2001). Inhibition of the LTP in dorsal and a facilitation in the ventral HC have been observed after acute stress exposure or in presence of physiological concentrations of CORT (Maggio and Segal, 2007, 2011). Moreover, evidence indicate that acute stress also impairs LTP through an increased cAMP-specific PDE4 (phosphodiesterase-4) activity (Chen et al., 2010). Given the need to normalize synaptic transmission and avoid LTP-inducing memory experiences that originate from synapsis activity saturation, a reduction in synaptic strength is fundamental to provide a functional synaptic model of memory. For this reason, LTP alone is insufficient and the intervention of LTD is required. Relating to the dynamic equilibrium between LTP and LTD, the presence of the same type of stress could be able to provoke opposite modifications, determining LTP impairment and LTD enhancement in the CA1 hippocampus through the activation of NMDA receptors (Kim et al. 1996). This dichotomous effect of stress on synaptic plasticity could be attributable to the development of learned helplessness by the exposed subjects, the reason why LTP or LTP-like alterations would occur and saturate hippocampal synapses circumventing the possibility to further sustain LTP when LTD activity has increased (Kim et al. 1996). Another possible occurrence is a "metaplastic" effect induced by stress, due to the imbalance in synaptic threshold for LTD over LTP induction (Abraham and Tate, 1997; Kim and Yoon, 1998). However, future studies are necessary to investigate more thoroughly signalling cascades at the basis of stress impact on LTP. A study related on a 30 min exposure of male rats to elevated maze has highlighted, immediately after the end of the stress protocol, an impairment in HPC of both LTP and pair pulse facilitation (PPF), this latter is linked to an enhancement in the amplitude of the second of two rapidly evoked excitatory postsynaptic potentials (EPSPs, Cazakoff and Howland, 2010). In the same work, the effect of the GR antagonist mifepristone, administered as pre-treatment in rats exposed to stress, was evaluated, demonstrating its efficacy in blocking stress-induced impairment both in PPF and LTP (Cazakoff and Howland, 2010). Moreover, a reduction in PPF after a single event of inescapable foot-shock stress was recorded through patch-clamp applied to pyramidal neurons in the medial PFC, indicating an increased probability of glutamate release (Musazzi et al., 2010).

1.8.2 CONSEQUENCES ON THE GLUTAMATERGIC SYSTEM

1.8.2.1 GLUTAMATE TRANSMISSION

Glutamate (Glu) is the most abundant excitatory neurotransmitter in the mammalian CNS and is involved in almost all the physiological functions of the brain. In neurons, it is mainly produced starting from glutamine (Gln) via a reaction catalyzed by glutamine synthase. Once synthesized, three different types of transporters (VGLUT1-3) contribute to its storage in the synaptic vesicles (Santos et al., 2009). Following the exocytotic release in the synaptic cleft, there are two main targets of Glu, namely ionotropic (iGlu) and metabotropic (mGlu) membrane receptors.

The first category includes NMDA, AMPA and kainate receptors which, unlike traditional ionotropic receptors, are not pentameric but tetrameric. Furthermore, each subunit presents only three transmembrane domains (TM1, 3 and 4) as TM2 does not completely cross the membrane (Traynelis et al., 2010; Karakas et al., 2015). They mediate the fast component of glutamatergic communication and are responsible for all the rapid changes that occur in neurons at the postsynaptic level. These receptors are permeable to Na^+ and Ca^{2+}, with a permeability profile that depends on the type of subunits they are composed of and on their possible combinations.

The second category, on the other hand, includes three groups of receptors (mGluI, mGluII, mGluIII), whose classification is based on the criteria of sequence homology, intracellular signalling pathways and pharmacological profile (Nicoletti et al., 2011). These are homo- or hetero-dimers with seven transmembrane domains coupled to G proteins, consisting of a long extracellular region at the N-terminal domain and a C-terminal portion facing the intracellular side. Group I includes mGlu1 and 5 receptors, group II includes mGlu2 and 3 receptors and group III includes mGlu4, 6, 7 and 8 receptors. Group I receptors are associated with G_q proteins, leading to the activation of phospholipase C and the production of the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG), with a consequent increase in intracellular Ca^{2+} and activation of protein kinase C (PKC). The receptors of groups II and III are coupled to Gi proteins, which exert an inhibitory action against adenylate cyclase, causing a decrease in intracellular levels of cAMP.

Once released in the synaptic cleft, Glu clearance is mediated by high-affinity excitatory amino acid transporters (EAATs), situated on surrounding glial cells (mainly EAAT1/GLAST and EAAT2/GLT-

1) and, on neurons (mainly EAAT3 and EAAT4) (O'Shea; 2002). Given the lack of degradative enzymes at the synapse level, Glu uptake represent the main mechanism to terminate its synaptic action. In glial cells, the enzyme glutamine synthetase (GlnS) catalyzes the formation of Gln from Glu. Gln is subsequently exported to the extracellular space and captured by glutamatergic nerve terminal, where is transformed back to Glu by the enzyme glutaminase (Erecinska and Silver, 1990). Astrocytes can also directly release this excitatory amino acid as a gliotransmitter by either Ca^{2+}-dependent vesicular exocytosis or by non-vesicular release (Araque et al., 2014; Woo et al.; 2012).

1.8.2.2 STRESS EFFECTS ON GLUTAMATE TRANSMISSION

In physiological conditions, Glu plays a fundamental role in synaptic plasticity, memory, and learning. However, in pathological circumstances it can give rise to harmful effects by acting as a powerful neuronal excitotoxin, thus triggering neurotoxicity (Sanacora et al., 2008). Enhancement of extracellular levels of Glu and activation of NMDA and non-NMDA ionotropic receptors, thus inducing high levels of intracellular calcium (Ca^{2+}), have been detected as consequence of stressful events. The excessive levels of the intracellular Ca^{2+} concentration imply a cascade of events leading to the damage of the cell membranes, involving caspase (proteases linked to the apoptotic process) and lipase activation. Another detrimental factor is represented by the induction of nitroxide synthase, which promotes the cellular levels of nitric oxide (NO), thus supporting the formation of reactive oxygen species (ROS), such as peroxynitrites and radicals of the hydroxyl ion, which strengthen the oxidative stress in the cell. Another cause of excessive ROS production is the induction of arachidonic acid synthesis, which in turn decreases Glu reuptake (McEwen, 1999; Sapolsky, 2000). The hyperactivation of ionotropic Glu receptors contributes to the neurotoxic effects of various acute events such as seizures and ischemia. Neurotoxicity, therefore, appears to be the response to the hyperstimulation of calcium-dependent enzymes and to the consequent production of oxygen free radicals (McEwen, 1999; Sapolsky, 2000).

1.8.2.3 STRESS EFFECTS ON GLUTAMATE RECEPTORS

Among the delayed or sustained effects of acute stress in the brain, the increase in NMDAR- and AMPAR- mediated synaptic currents in PFC pyramidal neurons represents one of the main responses at the postsynaptic level (Yuen et al., 2009, 2011).

This event, that strengthens Glu transmission, is closely related to the increase of the number of iGlu receptors at the postsynaptic plasma membrane operated by intracellular glucocorticoid receptors (Yuen et al., 2009, 2011). Rapid modifications induced by acute stress have been described in the hippocampal CA1 region through the action of membrane mineralocorticoid receptors, whose expression seems to be less represented in PFC (Karst et al., 2005; Olijslagers et al., 2008; de Kloet et al., 2005). Also, single stress session administration determines significative differences of Glu transmission in PFC compared to other brain areas (CA1, midbrain, NAc shell). In PFC, AMPAR and NMDAR increase their activity to a similar extent after acute stress or CORT exposure; whereas the other cerebral regions only AMPAR-mediated currents are selectively enhanced (Yuen et al., 2009, 2011; Karst and Joëls, 2005; Saal et al., 2003; Campioni et al., 2009). Another aspect of Glu receptors, connecting stress and glucocorticoids, regards their trafficking. Evidence testifying a facilitation of AMPARs recruitment or endocytosis, mediated by glucocorticoids, have highlighted an enhanced mobility of GluR2-containing AMPARs located to the surface of cultured hippocampal neurons after CORT exposure (Groc et al., 2008; Martin et al., 2009). Acute stress or a brief treatment with CORT have also proved to influence surface expression and synaptic density of NMDAR and AMPAR subunits, causing their significative increase in the PFC (Yuen et al., 2009, 2011). It is thought that the enhancement of glutamate receptors translocation from intracellular or extra synaptic surface pools towards the synaptic active zone of the membrane may support the synaptic potentiation determined by acute stress (Popoli et al., 2012).

The use of glucocorticoid receptor antagonists such as RU486, inhibitors of gene transcription such as actinomycin D or puromycin, or protein translation by anisomycin D extinguishes CORT delayed consequences at the excitatory postsynaptic level after short-term exposure, indicating the intervention of glucocorticoid receptors via their genomic pathway (Yuen et al., 2011). Among the earliest genes activated within this transduction pathway, those involved in coding of serum- and glucocorticoid-inducible kinases (SGKs) have been identified as key players in the enhancement of receptors recycling between early endosomes and the plasma membrane (van der Sluijs et al., 1992). These kinases operate the phosphorylation of the GDP dissociation inhibitor (GDI), allowing the formation of GDI–RAB4 complexes, whose main tasks include the facilitation of RAB4 cycle and the recycling of AMPARs to the synaptic membrane (Liu et al., 2010). In contextual fear conditioning, acute stress mediates inhibition of the mitogen activated protein kinase (MAPK) pathway in HC, an event that reduces the induction operated on the (early growth response protein 1 (EGR) via glucocorticoid receptor-induced genomic system (Revest et al., 2005). Short-term

exposure to stressful conditions modifies the expression of early genes related to excitatory synapses changes, the most influenced of which is the activity- regulated cytoskeletal-associated protein (Arc) with a specific increase in the PFC after acute restraint stress (Fumagalli et al., 2011).

1.8.2.4 STRESS EFFECTS ON GLUTAMATE RELEASE

Both acute and chronic stress lead to changes of basal Glu levels. An increase of the extracellular amount of this excitatory neurotransmitter has been detected in the limbic structures (cortex, HC, AG) after different types of stressful stimuli (Reznikov et al., 2007; Moghaddam, 1993).

In vitro exposure of hippocampal slices to CORT induced enhancement of the miniature excitatory postsynaptic potential frequency and decrease of paired-pulse facilitation in the pyramidal neurons of the CA1 hippocampal area, with a consequent higher probability of glutamate release (Karst et al., 2005). Previous studies using synaptosomes (isolated nerve endings) in superfusion have highlighted that exposure to foot shock, an acute stress protocol, also exerts effects on depolarization-evoked Glu release in the PFC and frontal cortex (Musazzi et al., 2010). The detected increase in stimulus-evoked release of this excitatory neurotransmitter could be attributable to modifications that take place inside the readily releasable pool of vesicles producing strengthening in the number of docked synaptic vesicles and their higher probability of being released (Rizzoli and Betz, 2005; Lonart and Sudhof, 2000). In particular, the acute stress induces fast enhancement of systemic corticosterone, which translates in the stimulation of membrane-located glucocorticoid receptors, leading in turn to a higher number of presynaptic SNARE protein complexes (Musazzi et al., 2010). As this change did not regard the number of complexes per vesicle, the most accredited mechanism mediated by acute stress may concern a rise of synaptic vesicles present in the RRP (Treccani et al., 2014). Interestingly, the in vitro exposure of synaptosomes to CORT for 20 min similarly increased the RRP but it was not linked to induction of glutamate release.

Electron microscopy stereology applied to medial PFC and the total internal reflection fluorescence microscopy carried out on synaptosomes validated the evidence that both acute stress and corticosterone enhances trafficking of synaptic vesicles towards the synaptic membrane (Treccani et al., 2014; Khanmohammadi et al., 2017). The identification of the pathway underlying the increase of RRP and vesicle mobilization triggered by both acute stress and in vitro CORT administration has been addressed through the blocking action of selective CORT receptor inhibitors, demonstrating

the involvement of the rapid, non-genomic, action at GR and MR specifically located on synaptic membranes (Treccani et al., 2014). Finally, the size of RRP has been shown to correlate with the degree of Synapsin I phosphorylation at Ser^9, whose levels increase in the presence of acute stressful events. Synapsin I, besides regulating the RRP, seems also to be implied in synaptic vesicle mobility and release probability under high-frequency stimulation (Musazzi et al., 2017).

1.8.2.5 STRESS EFFECTS ON GLUTAMATE CLEARANCE AND METABOLISM

Although studies related to stress effects in the brain have mainly focused on the neuronal component, emerging data have highlighted that stress can affect glial cells and in particular the functions related to glutamate clearance and metabolism, in which glia is strongly involved.

In the tripartite synapse, Glu transporters are located in strategic positions aimed at preventing the excitatory neurotransmitter spill over and the activation of extra synaptic receptors (Popoli et al., 2012). Accordingly, stimulation of peri- and extra synaptic NMDARs and mGluRs in the HC is regulated by the Glu uptake by glial glutamate transporters, whose action exerts also a small direct effect on synaptic AMPA-mediated excitatory postsynaptic potentials (Zheng et al., 2008). The excitotoxic damage of neurons can occur because of the regulation of EAAT2 expression and function, regulating activation of extra synaptic and synaptic NMDARs (O'Shea, 2002; Hardingham and Bading, 2010; Xu et al., 2009). As mentioned above, stress exposure triggers increase of Glu release, leading to $EAAT_s$ function modification, by reducing the clearance of excessive Glu in the extracellular space (O'Shea, 2002; Beart and O'Shea, 2007).

Post-mortem analysis of brain samples collected from patients suffering of mood disorders, mainly depression, have demonstrated a relevant decrease of glial cells numbers and density in PFC. These data have been confirmed by a reduction of the immuno-staining for the main intermediate filament protein in mature astrocytes, the glial fibrillary acidic protein (GFAP) in the same brain area and also in the AG and cerebellum (Ongur et al., 1998; Rajkowska and Miguel-Hidalgo, 2007; Miguel-Hidalgo et al., 2000; Altshuler et al., 2010). In rodent models, stress effects on glial cells have been mostly evaluated as consequence of chronic stress exposure, highlighting reduced proliferation of glial progenitor cells, diminished number of GFAP-positive cells and a lower expression of GFAP (Banasr et al., 2007; Banasr and Duman 2008). Instead, acute stress induced astrocytic hypertrophy in the neocortex (Murphy-Royal et al., 2020). a phenomenon leading to functional decoupling

between neurons and astrocytes, detectable monitoring the decrease of the typical astrocytic gap junction proteins connexin (Cx) 30 and 43 (Sánchez et al., 2020). The loss of appropriate astrocytic function, observed in response to stress, directly correlated with impairment of neuronal synaptic plasticity in PFC, responsible for the development of psychiatric-like symptoms in rodents (Murphy-Royal et al., 2020; Sun et al., 2012). Another recent study demonstrated the increase of GFAP expression as a consequence of reactive gliosis under specific stress conditions, like repeated restraint (Middeldorp and Ho, 2011; Kwon et al., 2011; Jang et al., 2008).

Modified Glu clearance and, consequently, altered glutamatergic neurotransmission do not directly correlate with alterations of GFAP expression in the brain of animals exposed to stress; nevertheless, complementary data have contributed to reveal the involvement of this astrocytic marker in glutamate uptake activity, through the modulation of transporter trafficking and surface expression (Hughes et al., 2004). Data obtained in synaptosomes purified from the FC and HC of rats exposed to short-term restrained stress, showed an enhancement of Glu uptake (Gilad et al., 1990). A subsequent investigation in hippocampal slices reported an increase of Glu uptake as immediate effect after FS exposure; whereas, reduction has been observed in HC, striatum and PFC 21 days after acute stress administration, indicating a biphasic time-dependent trend for Glu uptake in response to stress exposure (Almeida et al., 2010). EAAT2 function is strongly altered by stress, with repercussions on animal behaviour, and by glucocorticoids whose high levels increase the expression of the specific isoform GLT1b in the HC (Autry et al., 2006). Stress-mediated EAAT2-involving glutamate uptake modification also depend on processes affecting the highly conserved promoter sequences in the regulatory region of the transporter (Allritz et al., 2010). Specific synaptic EAAT2 trafficking, membrane stabilization, and clustering are essential to guarantee a proper neuronal activity through the regulation of genomic and non-genomic pathways (Nakagawa et al., 2008; Zhou and Sutherland, 2004). Moreover, lower expression of mRNA from the genes encoding for the glial Glu transporters, SCL1A2 (Solute Carrier Family 1 Member 2) and SCL1A3 (Solute Carrier Family 1 Member 3), has been detected in PFC and locus coeruleus during post-mortem analysis of patients suffering of major depressive disorders (Choudary et al., 2005; Bernard et al.; 2011). In the same context, a reduction of the expression of the gene related to GlnS has been described (Choudary et al., 2005; Sequeira et al., 2009). In rodents subjected to chronic unpredictable stress, the Glu-Gln cycle in the PFC was less effective, even if it did not base on the reduction of GlnS expression, thus indicating that other non-transcriptional elements can alter the stress-induced changes of glutamate metabolism (Banasr et al., 2010).

1.9 AN INTRODUCTION TO MOOD DISORDERS

Mood disorders have an impact on up to 40% of the population worldwide and within mental illnesses they represent the most frequently diagnosed form (Vahratian et al., 2021). Overall, mental disorders comprehend a group of psychiatric conditions, causing in the affected people marked alterations of mood, thoughts, perceptions, emotions, behaviour and feelings (Kohn et al., 2004; Ngui et al., 2010). The mood disorder with the highest prevalence (4,4%) of the world population is constituted by depression (GBD Disease and Injury Incidence and Prevalence Collaborators, 2016). In 2013, the Fifth Edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) introduced a new diagnostic category called “Trauma and Stress-Related Disorders” comprehending both adjustment disorders (ADs) and PTSD (American Psychiatric Association, 2013). This subdivision is functional because it allows clinicians to distinguish between normal (non-pathological) distress from acute, widespread, clinically elevated stress responses associated with AD and chronic psychiatric diseases (including PTSD). In particular, the symptom clusters established by the guidelines for diagnosing PTSD are represented by intrusion symptoms, persistent avoidance of stimuli, negative alterations in cognition and mood associated with the traumatic event, marked alterations in arousal and reactivity (American Psychiatric Association, 2013).

1.9.1 DEPRESSION

1.9.1.1 DIFFERENT FORMS OF DEPRESSION

The term “depressive disorders” (American Psychiatric Association, 2013) is referred to specific clinical entities and its use in clinical practice is more appropriate than the generic use of "depression". The Diagnostic and Statistical Manual of Mental Disorders (DSM) defines depressive disorders as a group of pathologies that have in common "the presence of a sad, empty or irritable mood, accompanied by somatic and cognitive changes that significantly affect the functioning ability of the individual". The pathologies included in this group differ in terms of duration, temporal distribution and etiology. In order to make a differential diagnosis between these disorders, the age of onset, duration of symptoms and any other conditions associated with them play therefore a crucial role.

Several forms of depression exist: major depressive disorder or single or recurrent episode (MDD), disruptive mood dysregulation disorder (DMDD), persistent depressive disorder (dysthymia), disorder due to medical-pharmacological conditions, depressive disorder with anxiety and premenstrual dysphoric disorder (Rush, 2007).

1.9.1.2 SYMPTOMS OF DEPRESSION

The chronic mental and physical alterations contribute to characterize the complexity and heterogeneity of depression from a clinical point of view. The most common symptoms include loss of energy, fatigue, difficulty in concentration and memory, agitation and nervousness, weight loss or gain, sleep disturbances (insomnia or hypersomnia), lack of sexual desire and physical pain. DSM-5 diagnostic criteria allow to diagnose depression in case of persistence of core cognitive, affective, strong-willed/motivational, behavioural, and physical symptoms for at least two weeks. Detailed symptoms are: altered appetite, altered sleep, psychomotor agitation (anxiety, irritability or rumination of thoughts), cognitive and physical dysfunctions, negative thoughts (feelings of hopelessness, helplessness, worthlessness, excessive guilt or self-hatred) and reiterative intentions of death and suicidal ideation. A peculiar feature of depressed patients is "anhedonia", defined as the marked loss of the ability to feel pleasure for almost all the activities that previously were able to stimulate interest in the subject, probably due to a dysfunction of the brain reward system (Hasler et al., 2004; Fawcett et al., 1983; Nestler et al., 2002; Pizzigalli, 2014). The lack of reactivity to any type of stimulus that derives from this phenomenon leads to moodiness, whose consequences permeate every aspect of life, compromising behaviour, social relationships, occupational life, and sense of well-being (Otte et al., 2016; American Psychiatric Association, 2013).

1.9.1.3 ETIOLOGY OF DEPRESSION

Depression clearly exhibits a leading psychiatric component, characterized by the related symptoms; it is therefore essential to trace its origins in the nervous system. The brain areas involved in the pathological processes that cause and/or are connected to depressive phenomena and other psychiatric diseases have been analyzed using the diagnostic imaging tool. Nevertheless, this technique did not prove to be exhaustive in immediately identifying the correlation between depressive phenomena and brain areas, since it is able to trace the activated area connected to depression, but not to understand the mechanism leading to the onset of depressive symptoms.

For over half a century, research oriented to deepen the pathophysiology of depression and to develop effective pharmacological treatments has been dominated by the monoamine hypothesis,

in which the deficiency of neurotransmitters such as NA and 5-HT was seen as the main contributor for the onset of the disease (Sanacora et al., 2012). In the last decades, this theory has been retained simplistic and is about to be outdated, especially due to the temporal discrepancy between the immediate effects of drugs on monoamine availability (minutes, hours) and their delayed therapeutic effects (several weeks). Indeed, novel diriment insights have introduced into the depression etiopathogenesis (Heninger et al., 1996; Hyman and Nestler, 1996; Hasler, 2010). Observations coming from preclinical literature focused on stress-based animal models of depression have highlighted a reduction of neurotrophic factors signaling, particularly of BDNF, and of the structural complexity in cortical and limbic brain areas (Duman and Monteggia, 2006; McEwen et al., 2016).

These data have been further supported by clinical studies conducted on the brain of depressed patients, in which a decrease of BDNF levels plays a primary role in the pathophysiology of the disease and in the definition of the "neurotrophic hypothesis" (Krishnan and Nestler, 2008; Castrén and Rantamäki, 2010; Autry and Monteggia, 2012). More recently, the "neuroplasticity hypothesis" has been introduced to integrate receptor intracellular signalling cascades with neurogenesis, gene expression, epigenetic alterations, synaptic mechanisms, and neurotrophin involvement. According to this theory, mood disorders would be caused by an impairment of the information processing within particular neuronal networks in the brain (Racagni and Popoli 2008; Nestler 2014). This theory predicts also that the most abundant transmitter in the brain, i.e. Glu, has a primary role in both the pathophysiology and pharmacological treatment of depression. (Sanacora et al., 2012; McCarthy et al., 2012; Musazzi et al., 2013). Nowadays, the prevailing hypothesis sustains that the pathophysiology of depression is not strictly caused by the dysregulation of a single neurotransmission mechanism, but it is influenced by multiple, simultaneously altered, systems (Sanacora et al., 2012; Musazzi et al., 2010).

1.9.1.4 THE GLUTAMATERGIC HYPOTHESIS

Several clinical studies have provided evidence of the involvement of the glutamatergic system in depression, starting from the preclinical finding that antagonism of the NMDA receptor could have a potential antidepressant effect (Trullas and Skolnick, 1990). As previously described, the expression of Glu receptors, glial GlnS and Glu transporters is altered in the brain of patients suffering from this disease, consistently with a dysregulation of Glu neurotransmission (Yüksel and Öngür, 2010; Niciu et al., 2014; Rajkowska et al., 1999; Choudary et al., 2005). In fact, limbic/cortical cerebral regions, cerebrospinal fluid and plasma of depressed individuals show abnormal amount of Glu and of its metabolites in association with brain volume and connectivity alterations (Küçükibrahimoğlu et al., 2009; Frye et al., 2007; Hashimoto et al., 2007; Yüksel and Öngür, 2010; Drevets, 2001; Koolschijn et al., 2009; Lorenzetti et al., 2009; Price and Drevets, 2010). The connection between modifications at the structural level and impaired mood and behaviour could base on the observation that HPC, PFC/FC, the regions most involved in structural changes, characterized by a volumetric reduction and rich of glutamatergic neurons/synapses, are the same implied in cognition, emotional behaviour, stress response and memory (Stockmeier et al., 2004; Price and Drevets, 2010; Drevets, 2001). These areas also play a regulatory function of synaptic homeostasis, whose role has been reported to be crucial in the pathophysiology of depression (Popoli et al., 2012; Duman and Aghajanian, 2012).

Acute and chronic stress conditions, as well as the prolonged depressive state, have been associated with neuronal atrophy and with a decrease in overall synaptic activity in the PFC and HC, alterations reproduced in other brain regions such as the AG and the NAc (Vyas et al., 2004; Bessa et al., 2013). It is thought that these synaptic changes derive from modified Glu release, caused by stressful situations, and from the consequent modifications of activity and connections also with glial cells. These modifications eventually lead to a deficitary production of neurotrophic factors and to a marked increase in circulating extracellular Glu. The excessive Glu implements mechanisms of excitotoxicity, determining modification of synaptic resistance and reducing the density of spines and dendritic branches in PFC (Krystal et al., 2013; Popoli et al., 2012). A further proposal concerns the imbalance deriving from the reduction of synaptic activity in PFC, which would lead to an enhanced functionality of other brain areas which are negatively modulated by the PFC, such as the AG, retained to be a cerebral region directly associated with anxious behaviours, and to a consolidation of the HPA cortex system (Savitz and Drevets, 2009).

In this light, the glutamatergic system acts as a primary contributor of the psychiatric pathology and a promising target for the therapeutic effect of antidepressant drugs (Sanacora et al., 2012).

1.10 PHARMACOLOGICAL TREATMENTS

The canonical approach to manage depression is based on the associated use of psychotherapy and pharmacological treatment with antidepressant drugs (Gelenberg et al., 2010; American Psychiatric Association, 2013; Cuijpers et al., 2014). This second aspect refers to the use of psychotropic molecules mainly acting at the CNS and characterized by heterogeneous chemical structure, pharmacokinetics, pharmacodynamics and biochemical action (Penn and Tracy, 2012).

In 1960s, the treatment of the disease has been revolutionized by the introduction of first-generation antidepressants, comprehending the tricyclic antidepressants (TCAs) and the monoamine oxidase (MAO) inhibitors (Nestler et al., 2002; Racagni and Popoli, 2008). The identification of their mechanism of action, attributable to an increase of the synaptic availability of monoamines through the block of synaptic reuptake and the reduction of catabolism, respectively, and of the unfortunate toxicological profile, has revealed the need to develop second-generation medications with better tolerability profile, i.e. the selective serotonin reuptake inhibitors (SSRIs) and the serotonin-noradrenaline reuptake inhibitors (SNRIs). The neuronal serotonin transporter (SERT) or the neuronal noradrenaline transporter (NET) are the selective targets of these molecules, allowing less toxicity and improving the safety profile (Millan, 2006; Rush et al., 2006). Subsequent research led to the introduction of drugs with additional effects on a variety of serotonin (5-HT) receptor subtypes. Two main representatives of this family are vilazodone, a partial agonist of the 5-HT1A receptor, and vortioxetine, whose action is exerted on several other 5-HT receptor subtypes (5HT1A, 5HT1B, 5HT1D, 5HT3, and 5-HT7). Another widely used class of antidepressants includes serotonin receptor type 2 antagonists (SARI), including trazodone and nefazodone, which are also able to exert a weak SERT inhibition. Among atypical antidepressants, mirtazapine is the most prescribed thanks to its combined block of α2-adrenergic autoreceptors and some 5-HT receptor subtypes (5HT2A and 5HT2C) by reducing negative feedback of catecholamine release (Cleare et al., 2015). A similar mechanism of action on 5-HT2C receptors has been described for agomelatine, which is also a melatonin receptor (MT1/MT2) agonist. The modifications that these drugs induce in the brain remain still unclear. It is, instead, well known the latency (at least several weeks) necessary for the therapeutic effect of antidepressants to be manifested, indicating that the strengthening of serotoninergic or noradrenergic neurotransmission is not responsible per se for the clinical outcome of these molecules (Nestler et al., 2002).

1.10.1 NEW THERAPEUTIC STRATEGIES

Although the development of SSRIs has significantly contributed to the improvement of the therapy for depression, there is still a need to implement the available therapeutic strategies, in particular to increase their effectiveness, reduce the latency, and decrease the number of patients who do not benefit from the existing treatments.

The glutamatergic hypothesis of depression represents one of the most popular starting points for the development of new therapeutic approaches. In fact, the ability of common antidepressants to reverse the process of neuronal atrophy is limited and requires a chronic treatment. On the contrary, drugs that target glutamatergic excitatory transmission, by acting on long-term synaptic plasticity, are potentially capable of producing rapid and profound alterations of neuronal circuits. The rapid actions triggered by glutamate involve the regulation of its ionotropic receptors, AMPA and NMDA, which affect learning processes, short- and long-term memory, accompanied by the modulation of synapse number and functionality and by the development of dendritic spines (Holtmaat and Svoboda, 2009). Recent studies have provided strong evidence that the Glu neurotransmission system can be an important and relevant target for new fast-acting antidepressants. In this context, the most widely studied compound at present is ketamine (Kadriu et al., 2019; Treccani et al., 2014).

1.10.2 KETAMINE

Recent studies have shown that the anesthetic drug ketamine, an antagonist of the NMDA receptors, is a promising active compound for the treatment of psychiatric diseases, such as treatment-resistant depression and obsessive-compulsive disorder (OCD; Murrough et al., 2013; Rodriguez et al., 2013). Thus, ketamine (KET) has attracted the attention of researchers as a possible antidepressant drug and several studies have shown its ability to induce a rapid (within 24 hours) and long-lasting (a few days) significant improvement of the depressive state (Berman et al., 2000; Diazgranados et al., 2010; Valentine et al., 2011; Zarate Jr et al., 2006; Murrough et al., 2013). In a later study, KET was found to provide almost immediate relief in bipolar patients with severe depression (Rodriguez et al., 2013). In both cases, the rationale for choosing this drug stemmed from the fact that inadequate regulation of the excitatory Glu receptors appears to play an active role in depression.

1.10.2.1 PHARMACOKINETICS AND PHARMACODYNAMICS OF KETAMINE

KET was originally developed in the 1960s as an anesthetic drug. As to the mechanism of action, KET is a non-competitive and non-subtype-selective antagonist of the NMDA glutamate receptor (Domino et al., 1965). From the chemical point of view, it is a liposoluble arylcyclohexylamine that rapidly crosses the blood-brain barrier and rapidly distributes also in other highly vascularized organs, such as liver and kidneys (Peltoniemi et al., 2016). KET primarily exists as a racemic mixture of two enantiomers: R (-) - KET and S (+) - KET (Mion and Villevieille, 2013). R (-) - KET shows potent and prolonged antidepressant properties in several preclinical studies; whereas S (+) - KET causes about four times NMDA receptor inhibition than the other enantiomer (Zanos et al., 2016; Yang et al., 2015; Moaddel et al., 2013). Following parenteral administration, KET shows low binding to plasma proteins and undergoes to a marked oxidative metabolism leading to 4-hydroxy-KET, 5-hydroxy-KET and norketamine (NK), an active metabolite which in turn is hydroxylated to 6-hydroxy-NK (HNK; Mion and Villevieille, 2013). The plasma peak is reached about 30 min after intravenous injection and metabolism involves liver, kidneys, intestine and lungs (Edwards and Mather, 2001; Mion and Villevieille, 2013). KET is characterized by an overall half-life of 2-3 hours, is excreted by glucuronide-conjugation in bile and urine. NK is eliminated more slowly, although its pharmacology is not yet well known (Mion and Villevieille, 2013).

KET is a non-competitive NMDA receptor antagonist that exerts its action through the binding to the "phencyclidine site", an allosteric intra-channel site (Veen et al., 2018). The NMDA receptor is an ion channel permeable to sodium (Na^{+}), calcium (Ca^{2+}) and potassium (K^{+}). The channel is blocked by magnesium ions (Mg^{2+}). To be opened, it must bind GLU and glycine at the same time and the membrane must be depolarized, to make the channel free from Mg^{2+}. KET binds to the cytoplasmic side of the receptor, inside the channel, and reduces its opening time, exerting a greater activity if the receptor has been previously activated by Glu (Zorumski et al., 2016). KET target not only the glutamatergic excitatory system being also able interfere with the inhibitory GABAergic system. Actually, at high concentrations it induces spinal analgesia by direct binding to spinal GABA receptors thus enhancing inhibitory transmission. Moreover, KET binds to the μ, δ, κ opioid receptors, although this interaction is not responsible for analgesia (Mion and Villevieille, 2013). The binding with κ receptors is linked to some psychic effects of KET. KET is also able to inhibit cholinergic receptors in the HC, striatum, and mPFC, causing psychic

phenomena (Mion and Villevieille, 2013). Furthermore, KET acts at the monoaminergic system by inhibiting the reuptake of NA, 5-HT and DA (Hancock and Stamford, 1999; Nishimura and Sato, 1999). Its activity at the dopaminergic system has provoked interest mainly for psychotic disorders, but it is also being investigated for its potential activity on mood disorders.

1.10.2.2 PHARMACOLOGICAL PROPERTIES

In 1960, the need of a safer anesthetic and analgesic drugs, compared to phencyclidine, led to the synthesis of KET, which, however, despite the better safety profile, showed a high potential for abuse due to its psychoactive properties (Domino et al., 1965; Mion e Villevieille, 2013). KET produces "dissociative anesthesia" characterized by a dreamlike state of detachment from own body and avulsion from the surrounding environment with visual and auditive disturbances, distorted perception of reality during the awakening phase (Corssen and Domino, 1966; Domino, 2010). KET is the only intravenous anesthetic that shows both analgesic properties and dose-dependent cardiovascular stimulation. It significantly increases heart rate, blood pressure and cardiac output within few minutes after intravenous administration (Sinner and Graf, 2008). In the CNS, KET produces increase of cerebral blood flow, oxygen consumption and intracranial pressure. Conversely, upper airways muscle tone and reflexes are maintained, although respiratory rate decreases (Sinner and Graf, 2008). The use of KET as an anesthetic in general surgery has been abandoned due to the appearance of post-operatory psychic phenomena (Liu et al., 2016). In the attempt to increase its efficacy and reduce undesirable effects, the individual isomers of the racemic mixture have been purified and S (+) KET seems to have greater anesthetic and analgesic properties in humans. However, this isomer also induces psychotomimetic effects (Berman et al., 2000). In 2019, subsequent studies led to the approval in the USA of intranasal esketamine as the first glutamatergic drug for the therapy of the treatment-resistant depression (TRD), even if the optimum dose, duration, and frequency of administration are not fully defined (Salahudeen M.S. et al., 2021).

The first evidence related to the potential use of KET as an antidepressant come from in-vitro studies conducted at the end of the 80s, which highlighted the inhibition of the binding of ligands to the NMDA receptor, with consequent reduction of ionic currents and of intracellular calcium levels (Sills and Loo, 1989; Reynolds and Miller, 1988; White et al., 1990; Cai e McCaslin, 1992). Pre-clinical and clinical studies showed that antidepressants are able to induce adaptive changes

of the NMDA receptor and that NMDA receptor antagonists mimic the behavioural effects of antidepressants (Trullas e Skolnick, 1990; Papp and Moryl, 1994; Papp and Moryl, 1996; Moryl et al., 1993; Meloni et al., 1993; Przegaliński et al., 1997; Vale et al., 1971; Crane, 1959). Most of the studies used a single 40 minutes i.v. infusion of (R, S)-KET the subanesthetic dose of 0.5 mg/Kg, leading to a significant symptomatic relief such as decrease of depressed mood, anhedonia and suicidal thoughts, as early as 2 hours after administration, in a high percentage (50-70%) of patients resistant to traditional therapies (Berman et al., 2000). These rapid effects, in contrast to traditional antidepressants, provided a cue for alternative hypotheses to the monoaminergic theory of depression (Ya-Ting et al., 2021).

The goal of the current studies is to identify the mechanism of action that supports the rapid antidepressant effects of KET for the development of novel fast-acting antidepressants without side effects, which currently limit the clinical use (Machado-Vieira et al., 2009; Aan Hen Rot et al., 2012; Krystal et al., 2013). The generally accepted model that justifies the therapeutic effect of KET in depression predicts the involvement of excitatory synapses (Panos and Todd, 2018). The mechanisms of action, namely, NMDA receptors inhibition, AMPA receptors over-expression, activation of synaptogenic signalling pathways (with the main contribution of BDNF, mTOR and eEF2) and the presence of the active metabolite HNK, allow this drug to act as an antidepressant (Panos and Todd, 2018).

1.10.2.3 EFFECTS ON GLUTAMATE TRANSMISSION

1.10.2.3.1 NMDA receptors inhibition

Several studies have been carried out to shed light on KET mechanisms involving the glutamatergic system, taking into account that this molecule, at sub-anesthetic doses, is able to trigger a fast transitory burst of extracellular Glu in PFC/FC (Moghaddam et al., 1997).

The main hypothesis formulated explain this unforeseen action of KET on Glu release concerns the NMDA receptors antagonism preferentially at tonically active inhibitory interneurons (Farber et al., 1998). The result of this blocking effect would be a reduction of the inhibitory control exerted by the GABAergic system, with consequent imbalance of the excitatory networks

(Abdallah et al., 2015; Duman et al., 2016). However, emerging data have questioned that the KET antidepressant effects are simply deriving from a block of NMDA receptors. The preclinical and clinical studies with NMDA antagonists (MK-801, Ro25-6981, memantine, AZD6765 and CP-101 606) or partial agonists of the binding site for glycine (D-cycloserine and rapastinel) did not show neither the strong, rapid and prolonged antidepressant effects of KET nor the presence of clinically relevant psychotomimetic or dissociative side effects (Kadriu et al., 2019; Zanos et al., 2016; Li et al., 2011; Maeng et al., 2008; Jiménez-Sanchez et al., 2014; Newport et al., 2015; Yang et al., 2015b). Although this molecule is the only NMDA receptor antagonist demonstrating antidepressant efficacy in multiple studies, according to the previous evidence, the blockade of NMDA receptors probably does not represent the only mechanism responsible for the clinical efficacy of KET. New investigations are therefore needed to clarify the contribution of NMDA receptor blocking in the therapeutic and side effects of KET (Aleksandrova et al., 2017).

1.10.2.3.2 AMPA receptors up-regulation

The studies carried out have confirmed the crucial role of AMPA receptors in the rapid antidepressant effects of KET (Park et al., 2015; Maeng et al., 2008; Koike and Chaki, 2014; Machado-Vieira et al., 2015). The rapid and transient increase of the release of Glu in the mPFC, induced by KET at subanesthetic doses, can in turn cause an acute activation of AMPA receptors and consequent facilitation of synaptic transmission (Chowdhury et al., 2016; Moghaddam et al., 1997). In rats, after treatment with KET, the increase of the binding of the drug to the hippocampal AMPA receptors has been observed, along with the increase in total or surface expression of the GluA1 and GluA2 subunits in the HC and PFC (Zanos et al., 2016; Koike and Chaki, 2014; Yang et al., 2016). Preclinical studies with the AMPA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-[f]-quinoxalina-2,3-dione (NBQX), induced a block of the KET rapid and prolonged antidepressant effects in the forced swim test (Koike et al., 2011; Zanos et al., 2016; Maeng et al., 2008; Koike and Chaki, 2014). Another evidence supporting the involvement of AMPA receptors in depression originates from fluoxetine and imipramine studies. The therapeutic mechanism of these drugs correlates with the upregulation of AMPA receptors in the HC and PFC and their antidepressant activity in the forced swim test is abolished by NBQX, indicating that AMPA receptor-mediated signalling facilitation could represent a convergence point downstream KET and classical antidepressants (Freudenberg et al., 2015; Du et al., 2007). These data indicate that the activation of AMPA receptors, is necessary for rapid or prolonged

effects of KET and encourage the investigation of drugs that are able to increase the function of the AMPA receptors for their potential utility in the treatment of depression (Aleksandrova et al., 2017).

1.10.2.3.3 Activation of synaptogenic signaling pathways

The most significant structural changes associated with depression are neuronal atrophy and loss of tissue volume in the HC and PFC (Duman and Aghajanian, 2012; Abdallah et al., 2016; Leuner and Shors, 2013). These aspects are supported by electrophysiological experiments performed in rat hippocampal slices which demonstrate that acute stress causes synaptic plasticity imbalance in favour of LTD versus LTP). This can in turn lead to synaptic hypofunction and neuronal loss. Also, chronic stress is known to reduce the density, length and number of dendritic spines in the HC and PFC (Duman et al., 2016; Wang et al., 2006; Xiong et al., 2004). These dysfunctions of synaptic structural plasticity seem to be reversed by KET (Li et al., 2011; Leuner and Shors, 2013; Kadriu et al., 2019). The main hypothesis assumes that several processes related to neuroplasticity act synergistically to induce the up-regulation of AMPA receptors and other synaptic proteins, which in turn increase synaptic strength and synaptogenesis (Duman and Li, 2012).

1.10.2.3.4 Ketamine and BDNF

Chronic stress in animals and depression in humans are associated with decreased BDNF expression and/or function in PFC and HC. Many studies have reported that KET causes increased translation and secretion of this neurotrophic factor (Schwartz et al., 2016; Yang et al., 2013; Zhou et al., 2014; Duman et al., 2016; Abdallah et al., 2015; Murakami et al., 2005; Bjorkholm and Monteggia, 2016). The disinhibition of glutamatergic neurons, due to the preferential inhibition of NMDA receptors expressed at GABAergic interneurons, leads to greater neuronal depolarization and increase of Glu release. The released Glu activates AMPA postsynaptic receptors, that increase the levels of Na^+ and Ca^{2+} in the cells. The high local intracellular concentration of Ca^{2+} triggers the vesicular release of BDNF (Jourdi et al., 2009). Following being released, BDNF binds to its TrkB receptor, which in turn activates the mTOR (mammalian target of rapamycin) pathway (Lepack et al., 2014; Lepack et al., 2016). Neurons are considered as the main source of BDNF, but the ability of astrocytes to synthesize this factor has recently been discovered, thus making astrocytes a pharmacological target for the treatment with antidepressants (Autry and Monteggia, 2012; Bjorkholm and Monteggia, 2016; Hisaoka-Nakashima et al., 2016). Indeed, SSRI and TCA

upregulate the BDNF mRNA via a monoamine-independent pathway in astrocytes but not in neurons (Allaman et al., 2011; Hisaoka-Nakashima et al., 2016; Kittel-Schneider et al., 2012; Musazzi et al., 2014; Takano et al., 2012). In this context, KET has shown to induce the suppression of BDNF astrocytic production, mitigating the ATP-evoked calcium signalling and reducing the number of exocytotic events at non-neuronal cell membranes (Stenovec et al., 2016).

1.10.2.3.5 Ketamine and mTOR

Another system implicated in the susceptibility of depression to KET is the mTOR signaling pathway, which regulates synaptic plasticity and translation of synaptic proteins. mTOR activation is accompanied by inhibition of the kinase of the Eukaryotic elongation factor 2 (eEF2) and the increase of synaptic protein synthesis. mTOR promotes synapse formation and maturation and its activity underlies the synaptogenic effects of KET (Zhou et al., 2014; Li et al., 2010; Ignacio et al., 2016). According to these evidence, both the antidepressant and synaptogenic effects of KET are blocked by pre-administration of rapamycin, a selective mTOR inhibitor. Activation of mTOR does not take place in presence of any class of traditional antidepressant drugs. Furthermore, consistently with the role of AMPA receptors in KET actions, the increase of mTOR phosphorylation and the activation of BDNF and GluR1 synthesis induced by KET are blocked by the AMPA receptor antagonist, NBQX (Li et al., 2010; Duman et al., 2016). Many studies reported in the literature agree that KET improves synaptogenesis and connectivity in the HC, PFC and associated regions through activation of different signalling pathways, including those involving BDNF and mTOR. Imaging studies of neural circuits in both animals and humans suggested that KET effectively counteracts the loss of connectivity between the PFC and other limbic structures in depressed individuals by activating these signal cascades. Thus, KET appears to re-establish adequate inhibitory control over HPA axis activation, negative emotions and anxiety (Abdallah et al., 2015; Duman et al., 2016).

1.10.2.3.6 The active metabolite hydroxynorketamine

KET is stereo-selectively metabolized into a wide range of metabolites, many of which are presumably inactive from a clinical point of view (Moaddel et al., 2013). It has been shown that HNK is the only active metabolite of KET, with similar efficacy but without the receptor binding properties of KET (Zanos et al., 2016). HNK reaches a brain concentration about 3 times higher in female rats than in males; this difference could be at the basis of the higher potency KET has in

females (Zanos et al., 2016; Zhou et al., 2014). This metabolite has shown a rapid and prolonged antidepressant action similar to that of KET; with a greater power of (R,R)-HNK enantiomer, reflecting the potency of the original compound. Similar to the results obtained with KET, the AMPA receptor antagonist NBQX, administered before the HNK injection, blocked the metabolite rapid and prolonged antidepressant effects (Zanos et al., 2016). HNK does not bind or inhibit NMDA receptors, but it causes a considerable increase of AMPA receptor-mediated synaptic transmission in the CA1 area of hippocampal slices, a rapid stimulation of the AMPA GluA1 and GluA2 subunit in hippocampal synaptosomes and increases BDNF levels 24 hours after treatment (Zanos et al., 2016).

2. AIM OF THE THESIS

Physiological stress is a response conserved throughout evolution and acts as an integral part of any adaptive biological system. It involves a wide range of effects, which from beneficial and protective features shift to deleterious consequences when environmental demand exceeds the body regulatory capacity.

Stress has been proven to exert a strong impact on cerebral functions, representing a risk factor for several diseases, such as neurodegenerative and psychiatric disorders (MD, anxiety and PTSD, De Kloet et al., 2005; Pittenger and Duman, 2008; Popoli et al., 2012; McEwen et al., 2015; Zannas et al., 2015). Moreover, the perception and the persistence of its consequences change from one person to another one based on the individual genetic background and/or to previous life experiences (Joëls et al., 2012; Koolhaas et al., 2011; Charney et al., 2004; Feder et al., 2009; Franklin et al., 2012; Russo et al., 2012). The stress response can also promote adaptive plasticity when physiologically activated and then inactivated, or maladaptive and harmful effects when the response is excessive or dysregulated (McEwen, 2005; Popoli et al., 2011). On this basis, it has been highlighted that most of the individuals are able to positively adapt to the environmental changes (resilients), whereas others cannot manage adaptation (vulnerables).

In this context, rodent models are very useful to study stress-related disorders, because they show maladaptive changes in the same brain regions altered in humans (Nestler and Hyman, 2010). It has been demonstrated that exposure to acute or subacute stress can induce not only rapid, but also sustained changes in synaptic function (Glu release, synaptic transmission/plasticity), neuroarchitecture (dendritic morphology, synaptic spines) and behaviour (cognitive functions) (Musazzi et al., 2010; Treccani et al., 2014; Musazzi et al., 2017). Moreover, neuroimaging studies have highlighted a volumetric reduction and a cytoarchitecture remodeling in psychiatric patient cortical and limbic brain areas, where glutamatergic neurons and synapses predominate (Savitz and Drevets, 2009).

An acute paradigm of FS stress has previously shown to quickly increase the glutamatergic transmission in rat PFC synaptosomes immediately after the stressful stimulus. These events are sustained by an increase of the ready release pool of synaptic vesicles, mediated by a non-genomic effect of mineral and glucocorticoid receptors, a phenomenon blocked by antidepressants (Musazzi et al., 2010; Treccani et al., 2014). Recent studies have demonstrated

that the glutamatergic homeostasis is also directly affected by glial cell impairment as a consequence of exposure to chronic stress (Tynan et al., 2013; Mayhew et al., 2015).

Despite the effects of re-exposure cycles to stressful events have been extensively deepened, the consequences of acute stress both in the short- and long-term are still poorly investigated. In this context, the role of non-neuronal cells in the differential response of resiliency or vulnerability to a single exposure to stress is still mostly unknown.

Starting on these premises, the main aim of this research project was to study, during time, behavioural, functional, and molecular alterations, induced by acute stress, in astrocytes in order to identify the factors that transform a physiological in a maladaptive stress response. At this purpose, we will study the anhedonic behaviour, a core symptom of depression and other stress-induced disorders at different time points from the stressful stimulus, to highlight a population developing maladaptive changes in the stress response (vulnerable, VUL) respect to resilient (RES) subjects (Nestler and Hyman, 2010; Christensen et al., 2011; Franklin et al., 2012). Then, we will analyze the glutamatergic transmission by measuring Glu release and its mechanisms from PFC neuronal (synaptosomes) and astrocytic (gliosomes) compartments of each phenotype. Finally, since recently KET has been demonstrated to be a valid antidepressant drug (Duman and Aghajanian, 2012), we will evaluate whether both functional and behavioural alterations induced by acute stress, will be affected by acute KET administration.

3. MATERIALS AND METHODS

3.1 ANIMALS

Male adult Sprague Dawley rats (250 - 300 g), purchased from Charles River, were used. Animals were housed in standard size cages (26 x 42 x 15 cm) at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light-dark cycle (lights on from 7 a.m. to 7 p.m.). Food standard diet obtained from Mucedola (Settimo Milanese, Milan, Italy) and water were freely available, except when required for sucrose and FS stress. All experimental procedures involving animals were performed in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC), approved by the Italian legislation on animal experimentation (Decreto Legislativo 26/2014) and authorized by the local ethics committee of the University of Genoa and the Italian Ministry of Health (DM140-14B – DGSAF24898). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

3.2 SUCROSE PREFERENCE TEST

Rats were isolated in clean cages (water and food usually present) and two bottles containing 1% sucrose solution were weighed and put in each cage for 2 hours, in order to habituate rats to drink sweet solutions. After 1 hour, bottles were inverted in their position, and, at the end of the two hours, animals were put again in their stabulation cages with free access to food and tap water. For the next 4-5 weeks (habituation period), the same animals were exposed for 1 hour, twice a week, to a 1% sucrose solution in parallel to tap water. In detail, animals were isolated in clean cages in the presence of one bottle containing the sucrose solution and one containing tap water, both previously weighed. After 30 min, the two bottles were inverted to eliminate side drinking preference. At the end, the two bottles were weighed again to measure sucrose and water consumption, to determine *sucrose preference* and *sucrose intake* (SI) to determine single rat *baseline* (that corresponds to the average of the sucrose solution assumed during the habituation period).

At the end of the habituation period, FS stress was applied and, at the different time points, rats were subjected to sucrose test in order to divide them in VUL (subjects with a reduction in SI

> 25% compared to the baseline) or RES (subjects with a reduction in SI less than 10%; Figure 3.1 and 3.2; see also Figure 4.1 in the Results section).

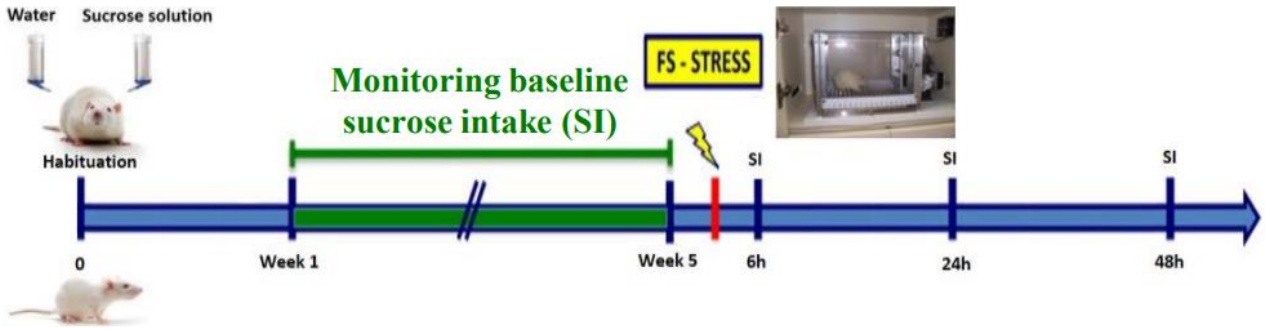

Figure 3.1: Experimental scheme to monitor SI at different time points after FS.

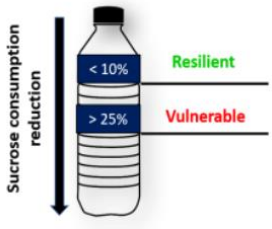

Figure 3.2: Representation of the criterion used to distinguish vulnerable and resilient animals based on SI.

Sucrose preference was calculated as: [sucrose solution intake (ml)/total fluid intake (ml)] x100] (Strekalova et al., 2011).

Total *sucrose intake* was defined as the sucrose consumption of each animal 6h, 24h or 48h after FS compared to each rat baseline during the 4-5 weeks of the habituation. SI was expressed as percentage of variation versus the respective baseline.

3.3 DRUG ADMINISTRATION

Animals were injected (i.p.) with a single acute administration of racemic KET (10 mg/Kg; MSD Animal Health Srl) 24h after FS. In parallel, physiological solution (0.9% w/v NaCl) was administered to controls.

3.4 FOOTSHOCK STRESS

The FS-stress was performed according to the following protocol: 40 min of FS-stress at 0,8 mA, 20 min of which were used to administer actual shock with random inter shock length between 2-8 sec. The FS-stress box was connected to a scrambler controller (LE 100-26, Panlab) that delivers intermittent shocks to the metallic floor. Sham-stressed rats (controls) were kept in the stress apparatus without delivering of shocks (Musazzi et al., 2010, Treccani et al., 2014, Bonanno et al., 2005). Rats were killed at different times (0h, 6h, 24h or 48h) after FS-stress and PFC were quickly dissected on ice and processed.

3.5 SERUM CORTICOSTERONE ASSAY

Within 1 hour from the sacrifice, blood was centrifuged at room temperature for 20 min at 3000g. Blood cells and coagulation factors were separated from the supernatant, which was divided into aliquots and stored at -80 °C. A commercial kit (Corticosterone EIA kit, Enzo Life Sciences Inc., Farmingdale, NY) was used to measure blood serum corticosterone levels (Musazzi et al., 2017).

3.6 WESTERN BLOTTING

Western blotting analysis was performed by using PFC synaptosomes, nuclear fraction or synaptosomal membranes, derived from controls, VUL and RES rats. Protein quantification was performed by BCA protein assay kit (Sigma-Aldrich, St. Louis, MO, USA). Appropriate amount (15-20 ug) of total proteins were separated by 10% SDS polyacrylamide precast gels (Bio-Rad, Hercules, CA, USA). Then, proteins were electrophoretically transferred to a Hybond p PVDF Transfer Membrane (GE Healthcare Life Sciences). Membranes were blocked for 60 min 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), β-actin 1:20000 (Sigma-Aldrich), and polyclonal antibody for phospho-synapsin I site 1 (Ser9) 1:1000 (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). Bands were detected and analyzed for optical density using an enhanced chemiluminescence substrate (ECL, LiteAblot PLUS, Euroclone, Milan, Italy), a chemiluminescence system (Alliance 6.7 WL 20M, UVITEC, Cambridge, UK), and UV1D software (UVITEC). Bands of interest were normalized for β-actin level in the same membrane.

3.7 GLIOSOMES AND SYNAPTOSOMES PURIFICATION

Rats were euthanized and the PFC rapidly removed. Tissue was homogenized in 14 vol. of 0.32M sucrose, buffered at pH 7.4 with Tris-HCl, using a glass-teflon tissue grinder (clearance 0.25 mm, 900 r.p.m.; Potter Elvehjem VWR International) and the homogenate was centrifuged (5 min, 1000g at 4°C) to remove nuclei and debris.

The supernatant was then centrifuged (5 min, 10000 r.p.m. at 4°C) and the pellet was resuspended in the 0.32M sucrose solution, gently stratified on a discontinuous Percoll® gradient (Sigma-Aldrich, St Louis, MO; 2, 6, 10 and 20% v/v in 0.32 M Tris-buffered sucrose) and centrifuged at 33,500g for 5 minutes. The layers between 2 and 6% (gliosomal fraction) and between 10 and 20% Percoll® (synaptosomal fraction) were collected, washed (20,000 x *g* for 15 min in physiological medium) and resuspended in physiological medium having the following composition: NaCl 140mM (VWR Chemicals); KCl 3mM (VWR Chemicals); $MgSO_4$ 1.2mM (Sigma Aldrich, Milan, Italy); NaH_2PO_4 1.2mM (Sigma Aldrich, Milan, Italy); $NaHCO_3$ 5mM (Sigma Aldrich, Milan, Italy); $CaCl_2$, 1.2mM (Sigma Aldrich, Milan, Italy); 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES) 10mM (Amresco Biochemicals); glucose 10mM (VWR Chemicals); pH 7.4.

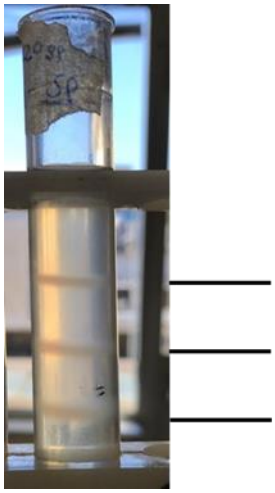

Figure 3.3: Representative image of the discontinuous Percoll gradient

Starting from the top:

interface between 2% and 6% of Percoll solution containing mainly purified gliosomes

interface between 6% and 10% of Percoll solution containing both gliosomes and synaptosomes

interface between 10% and 20% of Percoll solution containing mainly purified synaptosomes

3.8 NEUROTRANSMITTER RELEASE EXPERIMENTS

Gliosomes and synaptosomes were freshly prepared from homogenized PFC and aliquots were distributed on microporous filters placed at the bottom of a set of 24 parallel superfusion chambers maintained at 37 °C (Superfusion System, Ugo Basile, Comerio, Varese, Italy) and processed as previously described (Stigliani et al., 2006; Raiteri et al., 2008; Treccani et al. 2014; Tornese et al., 2019). Briefly, gliosomes and synaptosomes were resuspended in HEPES-buffered physiological medium and incubated (15 min, 37°C) with 0.05 μM [^{3}H]-D-Asp (PerkinElmer, Milan, Italy), a non-metabolizable analogue of Glu which labels the intra-terminal releasable pools of the excitatory amino acid (Fleck et al., 2001). Aliquots were distributed on 25 mm cellulose microporous filters (0.65 μm) (Millipore) placed at the bottom of a set of 24 parallel superfusion chambers maintained at 37°C (Figure 3.4). Superfusion was started with physiological medium at a rate of 0.5 ml/min and continued for 48 min. Gliosomes were exposed to a calcium free solution starting from min 20 of superfusion, to 10^{-7} M / 10^{-9} M TFB-TBOA (potent and selective glial Glu transporter EAAT1 and EAAT2 inhibitor; Tocris Bioscience, Bristol, UK) and to 10^{-5} M KB-R7943 (potent and selective inhibitor of the reverse mode of the Na^{+} /Ca^{2+} exchanger; Tocris Bioscience, Bristol, UK) starting from min 30 of superfusion, and maintained until the end of the experiment. Moreover, a 90 second depolarizing stimulus (15 mM KCl) was applied at min 39 of superfusion. Synaptosomes were only exposed to the 90 s 15 mM KCl stimulus. Samples were collected according to the following scheme: two 3-min samples (t= 36-39 and 45-48 min, basal release) before and after one 6-min sample (t= 39-45 min, stimulus-evoked release). Tritium released in each sample was calculated as fractional rate x 100 (percentage of the total synaptosomal tritium content at the beginning of the respective collection period). The stimulus-evoked overflow was estimated by subtracting the transmitter content in the two 3 min fractions, representing the basal release, from the 6 min fraction collected during and after the stimulation pulse. Appropriate controls were always run in parallel.

Figure 3.4: Image representing the superfusion system.

3.8.1 EXPERIMENTAL SCHEME OF RELEASE EXPERIMENTS

a) Immediately after FS

Immediately after FS, FS-rats and CTR were sacrificed without making any discrimination of the phenotype, as it was impossible at this time, and PFC was quickly dissected on ice and used for release experiments (Figure 3.5).

Figure 3.5: Graphic representation of the experimental scheme used to perform release experiments immediately after FS stress.

b) 6 hours after FS

6h after FS stress, sucrose preference test was applied to distinguish VUL and RES rats. In parallel, controls rats were sacrificed. PFC was quickly dissected on ice and used for release experiments (Figure 3.6).

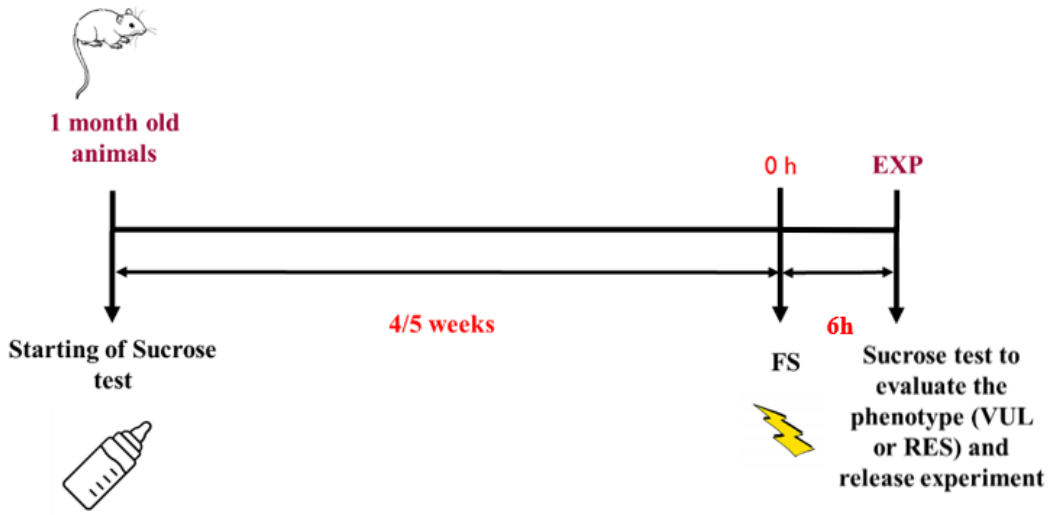

Figure 3.6: Graphic representation of the experimental scheme used to perform release experiments 6h after FS.

c) 24 hours after FS

24h after FS stress, sucrose preference test was applied to distinguish VUL and RES rats. In parallel, control rats were sacrificed. PFC was quickly dissected on ice and used for release experiments (Figure 3.7).

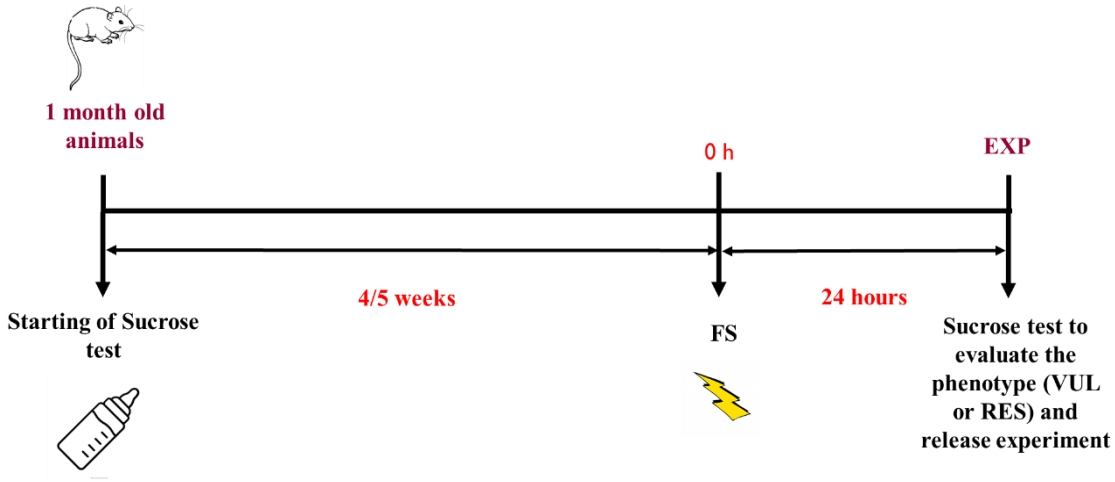

Figure 3.7: Graphic representation of the experimental scheme used to perform release experiments 24h after FS stress.

d) 48 hours after FS and 24 hours after KET treatment

24h after FS stress, sucrose test was applied to distinguish VUL and RES rats. In parallel, CTR rats were sacrificed. Half of the animals of each phenotype was treated with KET (10mg/kg i.p.) and the respective CTR with physiological solution. 48h after FS, phenotype evaluation was performed to analyze changes induced by KET. Then rats (VUL, RES, controls) were killed and PFC were quickly dissected on ice used for release experiments (Figure 3.8).

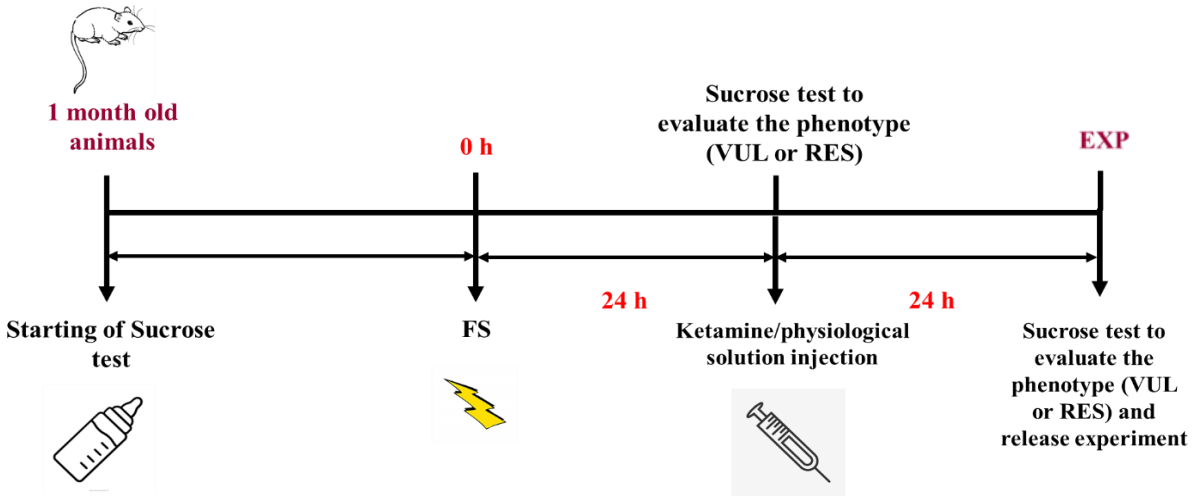

Figure 3.8: Graphic representation of the experimental scheme used to perform release experiments 48h after FS stress and 24h after KET injection.

3.9 STATISTICS

Data were expressed as mean ± SEM and p value < 0.05 was considered significant. Multiple comparisons were performed using the analysis of variance (one or two-way ANOVA) followed by Bonferroni post hoc test. Analyses were performed by means of SigmaStat (Systat Software, Inc., San Jose, CA, USA) software.

4. RESULTS

4.1 RESILIENT AND VULNERABLE PHENOTYPES ASSESSED BY SUCROSE INTAKE

The identification of the anhedonic phenotype induced by FS stress was performed by adapting the SI test, a paradigm traditionally used in models of chronic stress (Franklin et al., 2012). SI, defined as the percentage of glucose solution drunk by stressed animals compared to the baseline (the average volume of the glucose solution drunk during the 5 weeks preceding the sucrose test), was evaluated after exposing rats to FS stress.

On this basis, the evaluation of the SI, performed 24h after FS stress, showed a significant reduction in all the FS-rat population compared to controls (Figure 4.1, left panel, *p<0.05, FS-rats vs controls). Moreover, establishing a cut off corresponding to 25% reduction of SI respect to the baseline, FS-rats can be split in two different populations: one with a percentage of SI variation less than 25% respect to its baseline; the other with a percentage of SI reduction higher than 25%, (Figure 4.1, right panel, *p<0.001 VUL vs controls, #p<0.001 VUL vs RES). In our experiments, rats with at least 25% SI reduction were identified as VUL and those with less than 10% SI reduction were identified as RES. Moreover, only those rats showing an elevated preference (more than 80%) were used for experimental procedures.

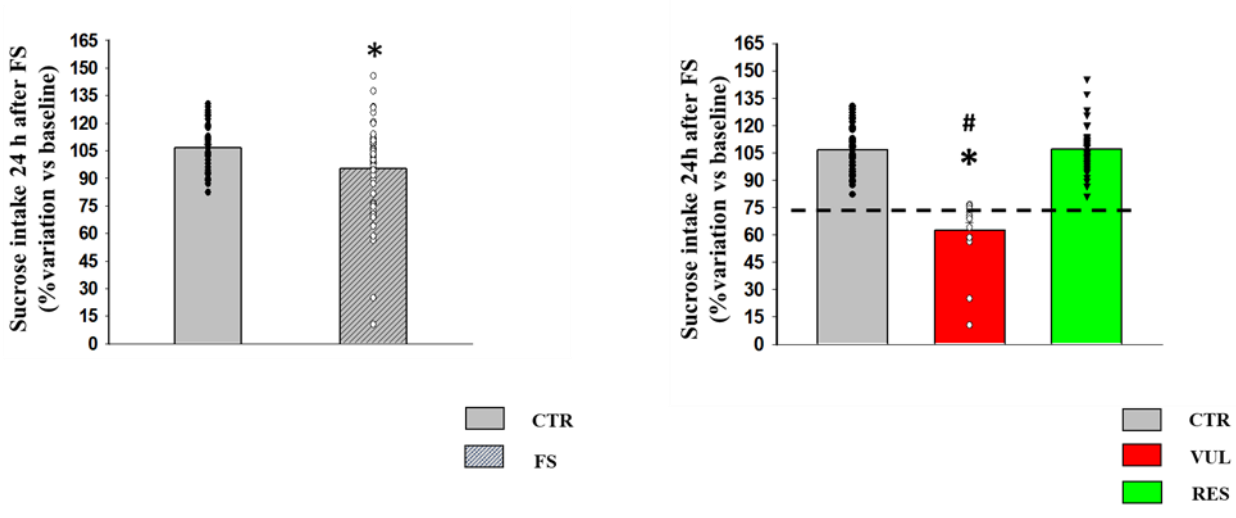

Figure 4.1. VUL and RES phenotype assessed by SI in FS-rats.
Left panel: SI, expressed as % variation respect to the SI baseline, was measured 24h after FS in stressed rats and controls. Data are expressed as MEAN ± s.e.m of 47 controls and 64 FS-rats. One-way ANOVA followed by Bonferroni post-hoc test. *p<0.05 vs controls.

Right panel: SI, expressed as % variation respect to the SI baseline, was measured 24h after FS in controls, VUL and RES rats. Data are expressed as MEAN ± s.e.m of 47 controls, 17 VUL and 47 RES rats. One-way ANOVA followed by Fisher's LSD post-hoc test. *p<0.001 vs controls; #p<0.001 vs RES rats.
The number of animals considered for each phenotype is based on a representative sample extrapolated from the total rat amount.

4.2 ANHEDONIC BEHAVIOUR IN FS-RATS DURING TIME

To investigate short- and long-term pathophysiological alterations induced by FS stress in VUL and RES rats, separately, SI was measured at different time points (6h, 24h and 48h) after FS stress. As shown in Figure 4.2, the anhedonic phenotype already occurred 6h after FS stress and lasted till 48h, the maximum time point investigated. More in detail, the data shown a significant reduction of SI in VUL rats at all the three time points compared to both control and RES rats (*p<0.001 vs controls, #p<0.001 vs RES). An unexpected significant SI increase was found in RES animals compared to controls at 24h after FS (*p<0.001 vs controls).

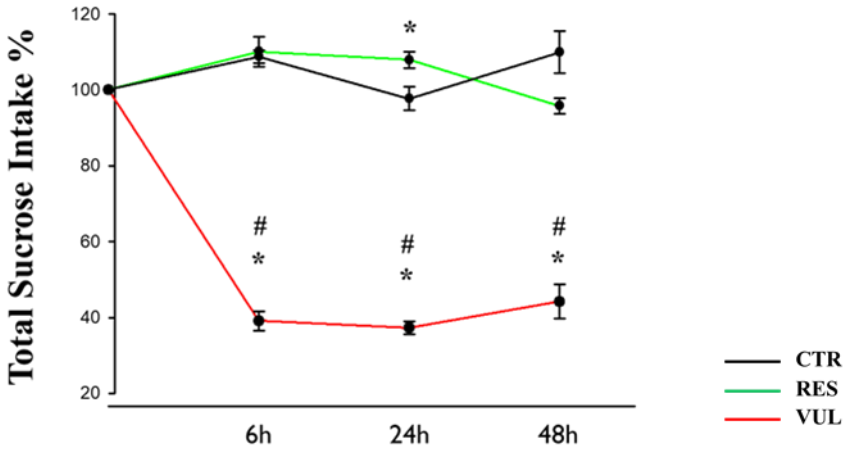

Figure 4.2. Sucrose intake in VUL and RES rats at 6h, 24h and 48h after FS stress.
SI was measured 6h, 24h and 48h after FS in control, VUL and RES rats and expressed as % variation respect to the SI baseline. Data are expressed as MEAN ± s.e.m. of 19 controls, 39 VUL and 16 RES at 6h after FS stress; 58 controls, 65 VUL and 57 RES at 24h after FS stress; 12 controls, 9 VUL and 3 RES at 48h after FS stress. One-way ANOVA followed by Bonferroni post hoc test. *p<0.001 vs controls; #p<0.001 vs RES rats.

4.3 BEHAVIOURAL PHENOTYPE DISTRIBUTION AT 6H, 24H AND 48H AFTER FS

The analysis of the phenotype distribution during time showed that: i) 6h after FS, VUL rats were 70.18% and RES 29.82% over a total of 57 rats; ii) 24h after FS, VUL rats were 53.85% and RES 46.15% over a total of 129 rats; iii) 48h after FS, VUL were 72,73% and RES 27,27% over a total of 11 (Figure 4.3).

These data suggest that a portion of VUL rats at 6h spontaneously switched to a RES phenotype at 24h, thus supporting the hypothesis that plastic modifications can increase resilience over time after FS stress. Data at 48h after FS stress do not reflect a stability in the phenotype distribution compared to 24h, as one could have expected, and this is probably due to the low number of animals analyzed till now at this last time point.

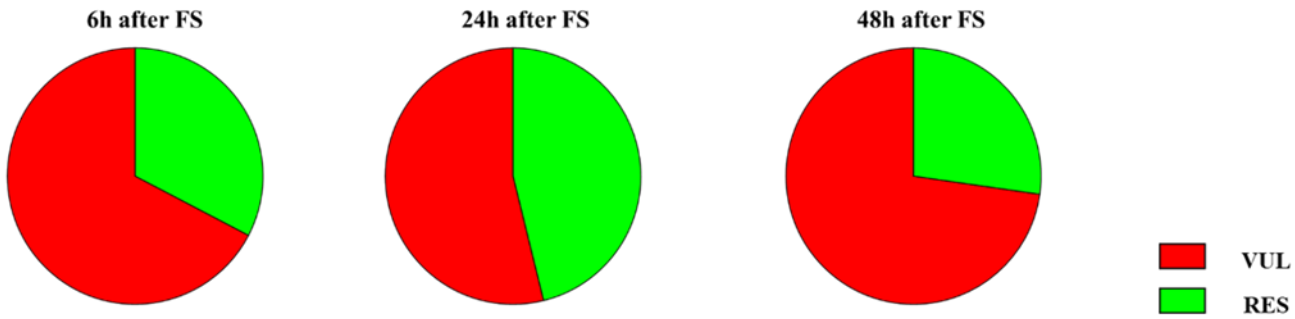

Figure 4.3. Percentage distribution of VUL and RES rats at 6h, 24h and 48h after FS stress.
VUL and RES rats have been identified by evaluating SI after 6h, 24h and 48h from FS (57 rats at 6h, 129 rats at 24h and 11 rats at 48h).

4.4 CORTICOSTERON LEVEL AND MINERAL AND GLUCOCORTICOID RECEPTOR EXPRESSION IN VUL AND RES RATS AT 24H AFTER FS

In previous studies, it has been demonstrated that the FS stress protocol induced an increase in circulating corticosterone (CORT) levels in FS-rats immediately after the stressful stimulus (Treccani et al., 2014). Interestingly, at variance from the alteration of the anhedonic phenotype shown above, the CORT peak undergoes normalization already 2 hours later its immediate onset (Musazzi et al., 2016). Our present data demonstrated that CORT serum level was not modified 24h after FS also considering RES and VUL animals separately respect to controls (Fig. 4.4).

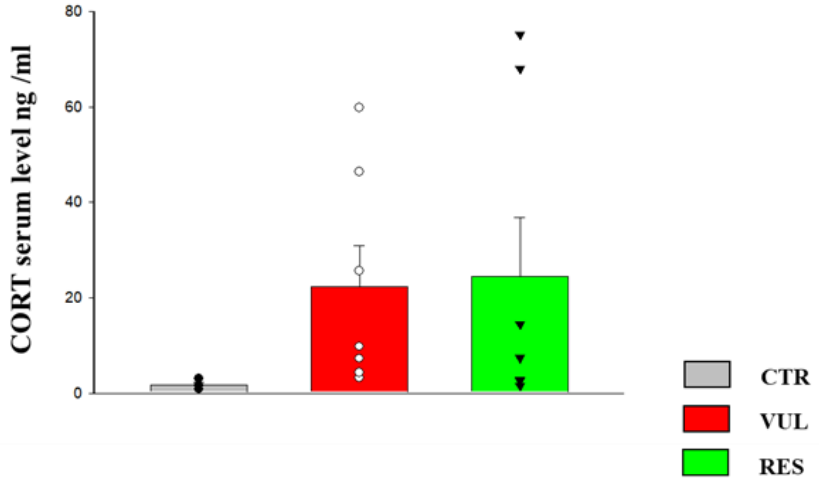

Figure 4.4. CORT level in VUL and RES rats at 24h after FS stress.
CORT level has been measured VUL and RES rats respect to controls at 24h after FS stress. Data are expressed as MEAN ± s.e.m. of 4 controls, 7 VUL and 7 RES rats. One-way ANOVA followed by Bonferroni post hoc test.

Moreover, the expression level of both mineral corticoid (MRs) and glucocorticoid (GRs) receptors, known to be overexpressed in PFC synaptosomes of FS-rats immediately after FS stress (Treccani et al., 2014), was here investigated in PFC nuclear fraction of VUL and RES animals 24h after FS stress. Our data showed that MRs were overexpressed in RES rats and GRs in VUL rats, compared to controls (Fig. 4.5, panel a and b). Instead, the phosphorylated form of GRs did not change in VUL and RES rats (Figure 4.5, panel c). These data suggest a different contribution of these two receptors to VUL and RES phenotypes.

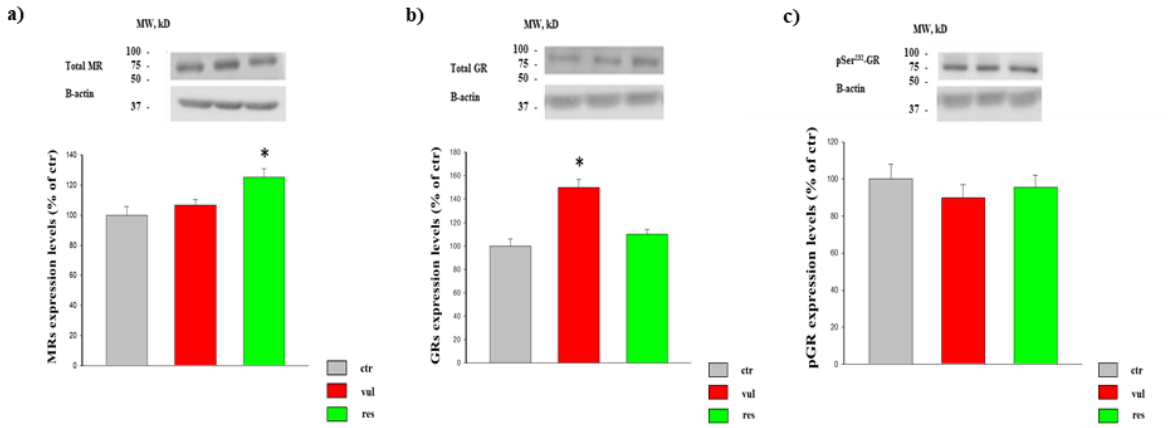

Figure 4.5. MRs and GRs expression in PFC nuclear fraction of VUL and RES rats at 24h after FS stress. Expression of MRs (a), GRs (b) and pSer^{232}-GR (c) in PFC nuclear fraction of VUL and RES rats respect to controls at 24h after FS is reported. Proteins were determined by SDS-PAGE and Western blotting using selective antibodies (see the Methods section). Representative immunoreactive bands are reported above the respective quantification graph. Data are MEAN ± s.e.m of 10-13 independent experiments. One-way ANOVA followed by Bonferroni post hoc test. *$p < 0.05$ versus controls.

4.5 EFFECTS OF ACUTE STRESS ON GLUTAMATE RELEASE IN PFC GLIOSOMES OF VUL AND RES RATS

To assess whether the different anhedonic phenotypes could be paralleled by functional modifications, we investigated the release of Glu from PFC gliosomes in VUL and RES rats at different time points after FS stress.

4.5.1 BASAL AND 15mM KCl-EVOKED GLUTAMATE RELEASE FROM PFC GLIOSOMES IN FS-RATS IMMEDIATELY AFTER FS

Release experiments were conducted immediately after FS stress in synaptosomes and gliosomes without distinguishing the VUL or RES phenotype. As reported in Figure 4.5.1, no change of basal and 15mM KCl-evoked [^{3}H]D-Asp release was observed in PFC gliosomes from FS-rats immediately sacrificed after FS stress compared to controls. Similar results were also obtained in absence of calcium in the physiological medium. In previous studies with synaptosomes obtained from the same brain region, it was demonstrated that the 15 mM-evoked Glu release was significantly increased in the stressed group compared to controls, whereas no differences were found in basal release (Musazzi et al., 2010).

These data suggest that no alterations take place in PFC gliosomal Glu release immediately after FS and point out that acute stress immediately affects Glu release only in synaptosomes.

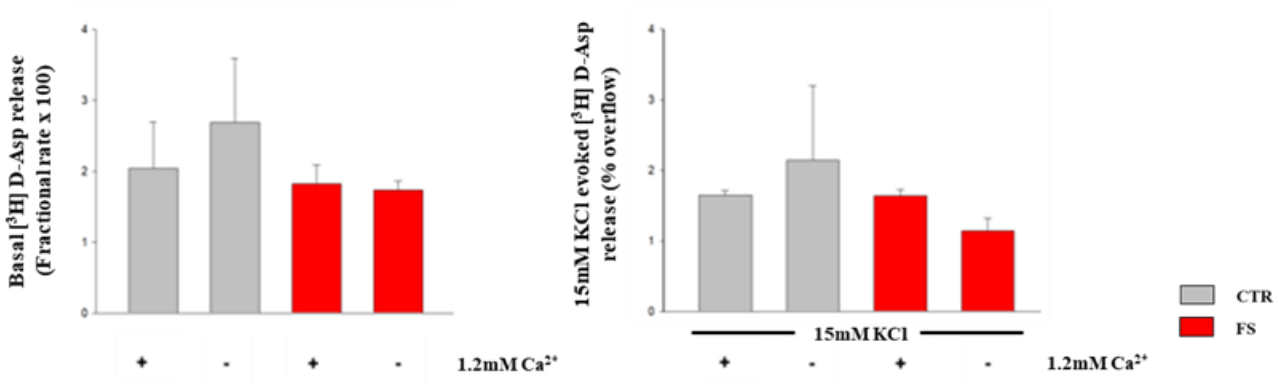

Figure 4.5.1. Basal (left) and 15mM KCl-evoked [^{3}H]D-aspartate release (right) from PFC gliosomes in stressed-animals and controls immediately after FS stress.
Animals were immediately sacrificed after the FS stress session. Gliosomes were prepared as described in the Methods section and exposed in superfusion to a 90s 15 mM KCl depolarizing stimulus in presence or absence of Ca^{2+}. Basal release (left panel) was expressed as the content of [^{3}H]D-Asp in the sample collected t=36-39 min of superfusion, before the introduction of the depolarizing stimulus, and are expressed as fractional rate percent. The net amount of stimulus-released neurotransmitter (right panel) was calculated by subtracting the transmitter content of the two 3-min basal release fractions (36-39 min and 45-48 min) from the release evoked

in the 6-min sample collected during and after the depolarization pulse (39-45 min, stimulus-evoked release). Data are expressed as MEAN ± s.e.m of 4 independent experiments. Two-way ANOVA.

4.5.2 BASAL AND 15mM KCl-EVOKED GLUTAMATE RELEASE FROM PFC GLIOSOMES IN VUL RATS 6H AFTER FS.

To verify whether the onset of the anhedonic phenotype detected 6h after FS was paralleled by functional modifications, release experiments were performed at this time point to check PFC gliosomal Glu release in VUL rats respect to controls. As reported in Figure 4.5.2, no changes of basal or 15mM KCl-evoked [^{3}H]D-Asp release was observed in PFC gliosomes in VUL rats compared to controls. Moreover, a significant reduction of the stimulus-evoked [^{3}H]D-Asp release was observed in the absence of calcium in VUL animals.

These data suggest that no alterations are present in PFC gliosomal Glu release 6h after FS stress.

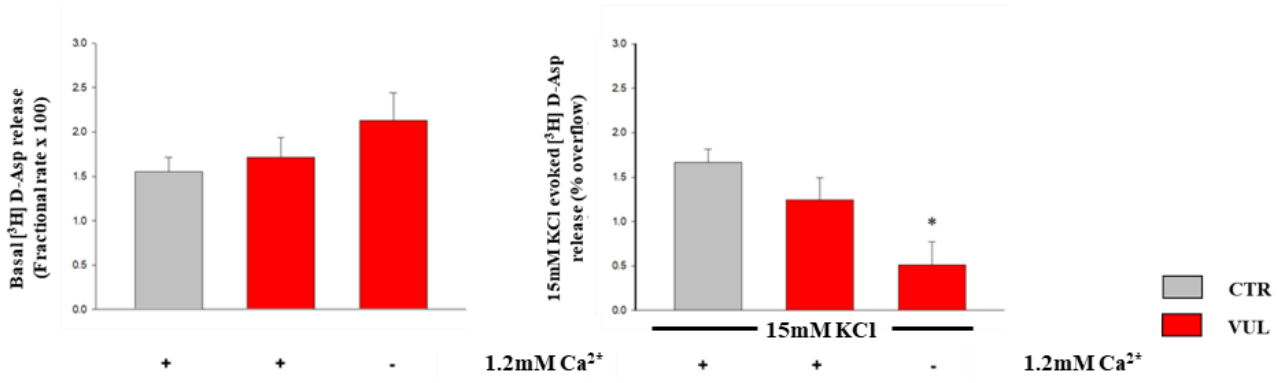

Figure 4.5.2. Basal (left) and 15mM KCL-evoked [^{3}H]D-aspartate release (right) in PFC gliosomes in VUL and controls animals 6h after FS stress.
VUL and control animals were sacrificed 6h after the FS-stress session. Gliosomes were prepared as described in the Methods section and exposed in superfusion to a 90s 15mM KCl depolarizing stimulus in presence or absence of Ca^{2+}. Basal release (left panel) was expressed as the content of [^{3}H]D-Asp in the sample collected t=36-39 min of superfusion, before the introduction of the depolarizing stimulus, and are expressed as fractional rate percent. The net amount of stimulus-released neurotransmitter (right panel) was calculated by subtracting the transmitter content of the two 3-min basal release fractions (36-39 min and 45-48 min) from the release evoked in the 6-min sample collected during and after the depolarization pulse (39-45min, stimulus-evoked release). Data are expressed as MEAN ± s.e.m of 8 independent experiments. One-way ANOVA followed by Bonferroni post hoc test. *p<0.05 versus controls.

4.5.3 BASAL AND 15 mM KCl EVOKED GLUTAMATE RELEASE FROM PFC GLIOSOMES 24H AFTER FS IN VUL AND RES RATS

A set of release experiments was performed 24h after FS stress. PFC gliosomes, obtained from controls, RES and VUL rats at 24h after FS stress, were exposed to a 90s 15mM KCl depolarizing stimulus. No differences were observed in the basal [^{3}H]D-Asp release, in both VUL and RES respect to control rats (Figure 4.5.3 left). Conversely, exposure to 15mM KCl induced a significant increase (30%) of [^{3}H]D-Asp release in PFC gliosomes obtained from VUL animals compared to both RES and control rats, whereas no difference was detected in RES rats compared to controls (Figure 4.5.3 right).

Differently from the previous two time points, these data show that 24h after FS the depolarizing stimulus-evoked [^{3}H]D-Asp release was significantly increased in PFC gliosomes in VUL rats only.

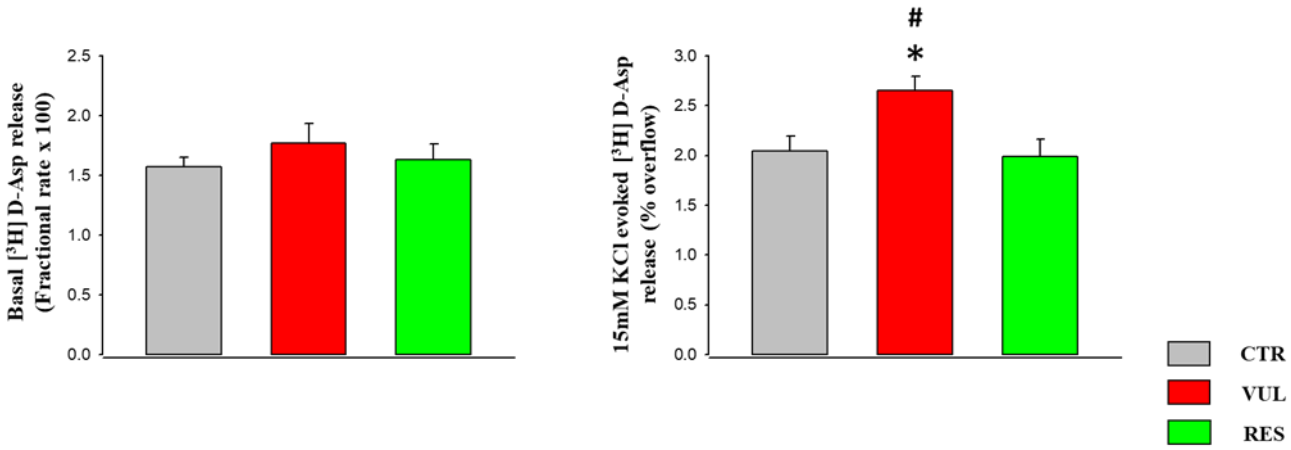

Figure 4.5.3. Basal (left) and 15mM KCl-evoked [^{3}H]D-aspartate release (right) in PFC gliosomes in VUL, RES and controls animals 24h after FS stress.
VUL, RES and control animals were sacrificed 24h after the FS-stress session. Gliosomes were prepared as described in the Methods section and exposed in superfusion to a 90s 15mM KCl depolarizing stimulus. Basal release (left panel) was expressed as the content of [^{3}H]D-Asp in the sample collected t=36-39 min of superfusion, before the introduction of the depolarizing stimulus, and are expressed as fractional rate percent. The net amount of stimulus-released neurotransmitter (right panel) was calculated by subtracting the transmitter content of the two 3-min basal release fractions (36-39 min and 45-48 min) from the release evoked in the 6-min sample collected during and after the depolarization pulse (39-45min, stimulus-evoked release). Data are expressed as MEAN ± s.e.m of 10-18 independent experiments. One-way ANOVA followed by Bonferroni post-hoc test. *$p<0.05$ vs controls; #$p<0.05$ vs RES.

4.5.4 MECHANISMS SUPPORTING THE INCREASED GLUTAMATE RELEASE FROM PFC GLIOSOMES 24H AFTER FS IN VUL RATS

A set of experiments was carried out in order to investigate the mechanisms underlying the augmentation of the 15mM KCl-evoked [^{3}H]D-Asp release present in PFC gliosomes 24h after FS stress in VUL animals. To this purpose, gliosomes were exposed to the following experimental conditions: i) the absence of calcium in the physiological medium, ii) the presence of 0.1 µM TFB-TBOA (a potent and selective inhibitor of the glial Glu EAAT1 and EAAT2 transporters), or iii) the presence of 10µM KB-R7943 (a selective inhibitor of the Na^+/Ca^{2+} exchanger when operating in the reverse mode). As reported in figure 4.5.4, the basal release of [^{3}H]D-Asp did not show any difference between VUL and controls in any of the condition analyzed. On the other side, the 15mM KCl-evoked [^{3}H]D-Asp release was practically abolished by 0.1µM TFB-TBOA, both in VUL (**p<0.01 versus controls) and controls (***p<0.001 versus controls) animals. The calcium-free solution as well as 10µM KB-R7943 did not modify the stimulus-evoked [3H]D-Asp release neither in VUL nor in controls rats, thus minimizing the occurrence of exocytotic release mechanisms, as well as the involvement of the Na^+/Ca^{2+} exchanger operating in a reverse mode.

These data suggest that the excessive [^{3}H]D-Asp release induced by 15 mM KCl in PFC gliosomes, present in VUL rats 24h after FS stress, is mainly mediated by the Glu transporters when working in the in-out mode.

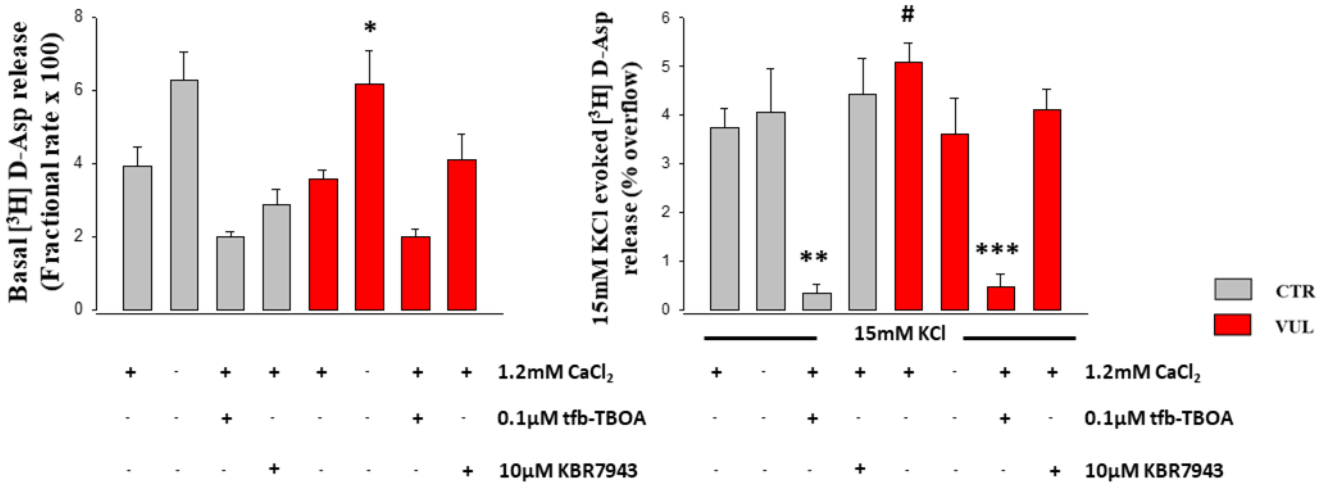

Figure 4.5.4. Basal (left) and 15mM KCl-evoked [^{3}H] D-aspartate release (right) in PFC gliosomes in VUL and controls animals 24h after FS stress.
The mechanisms underlying the increased evoked [3H]D-aspartate release were studied in VUL and controls animals 24h after FS. Animals (controls and VUL) were sacrificed 24h after the FS-stress session. Gliosomes

were prepared as described in the Methods section and exposed to different experimental conditions (0.1µM TFB-TBOA or 10µM KB-R7943) added to physiological medium (MP) or to a Ca^{2+}-free solution after 30 min of superfusion and maintained until the end of the experiments. Basal release (left panel), was expressed as the content of [^{3}H]D-Asp in the sample collected t=36-39 min of superfusion, before the introduction of the depolarizing stimulus, and are expressed as fractional rate percent. The net amount of stimulus-released neurotransmitter (right panel) was calculated by subtracting the transmitter content of the two 3-min basal release fractions (36-39 min and 45-48 min) from the release evoked in the 6-min sample collected during and after the depolarization pulse (39-45min, stimulus-evoked release). Data are expressed as MEAN ± s.e.m. of 3-5 indipendent experiments. One-way ANOVA followed by Bonferroni post-hoc test. **p<0.01 and ***p<0.001 vs controls, #p<0.05 vs not FS controls.

4.6 BASAL AND 15 mM KCl-EVOKED GLUTAMATE RELEASE FROM PFC SYNAPTOSOMES 24H AFTER FS STRESS IN VUL AND RES RATS

Previous studies demonstrated that acute FS stress increased only the depolarization-evoked [^{3}H]-D-Asp release from PFC purified synaptosomes in FS-rats respect to controls (Treccani et al., 2014). Moreover, the increased Glu release, occurring immediately after FS stress, lasts for at least 24h (Musazzi et al., 2016). Here we studied the basal and the 15mM-evoked [^{3}H]-D-aspartate release from PFC synaptosomes 24h after FS stress, distinguishing VUL and RES phenotypes.

As reported in Figure 4.6, the basal release of [^{3}H]-D-Asp was significantly increased only in VUL rats compared to the controls, whereas the 15mM KCl-evoked [^{3}H]-D-aspartate release was enhanced in both RES and VUL animals respect to the controls.

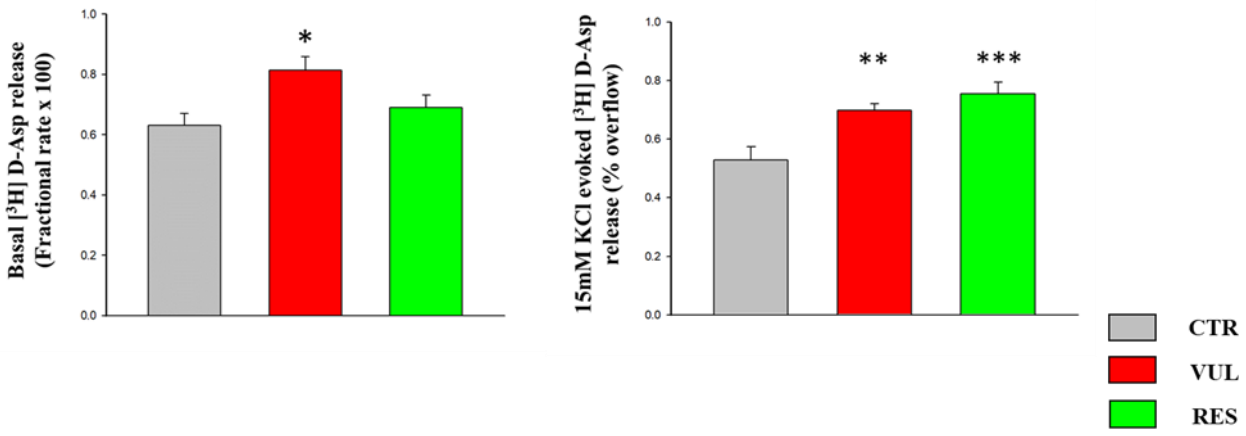

Figure 4.6. Basal (left) and 15mM KCl-evoked [^{3}H]D-aspartate release (right) in PFC synaptosomes in VUL, RES and controls animals 24h after FS stress.
VUL, RES and control animals were sacrificed 24h after the FS-stress session. Synaptosomes were prepared as described in the Methods section and exposed in superfusion to a 90s 15mM KCl depolarizing stimulus. Basal release (left panel) was expressed as the content of [^{3}H]D-Asp in the sample collected t=36-39 min of superfusion, before the introduction of the depolarizing stimulus, and are expressed as fractional rate percent. The net amount of stimulus-released neurotransmitter (right panel) was calculated by subtracting the transmitter content of the two 3-min basal release fractions (36-39 min and 45-48 min) from the release evoked in the 6-min sample collected during and after the depolarization pulse (39-45min, stimulus-evoked release). Data are expressed as MEAN ± s.e.m of 10-13 independent experiments. One-way ANOVA followed by Bonferroni post-hoc test. *p<0.05, **p<0.01 and *** p<0.001.

4.6.1 MECHANISMS SUPPORTING THE INCREASED GLUTAMATE RELEASE FROM PFC SYNAPTOSOMES 24H AFTER FS IN VUL AND RES RATS

To unveil the molecular mechanisms sustaining the excessive Glu release found in PFC synaptosomes, the expression of the total Synapsin I, a protein involved in the readily releasable pool (RRP) formation and in neurotransmitter release, as well as of its phosphorylated form at Serin in site 9, were assessed 24h after FS in VUL and RES rats. It was observed that the total expression of Syn I was not modified in PFC synaptic membranes from both VUL and RES rats compared to controls; whereas, its phosphorylated form was significantly increased in both phenotypes, thus indicating an overactivation of Synapsin I (Figure 4.6.1, *p<0.05; **p<0.01).

These data are in line with the excessive Glu release observed in PFC synaptosomes.

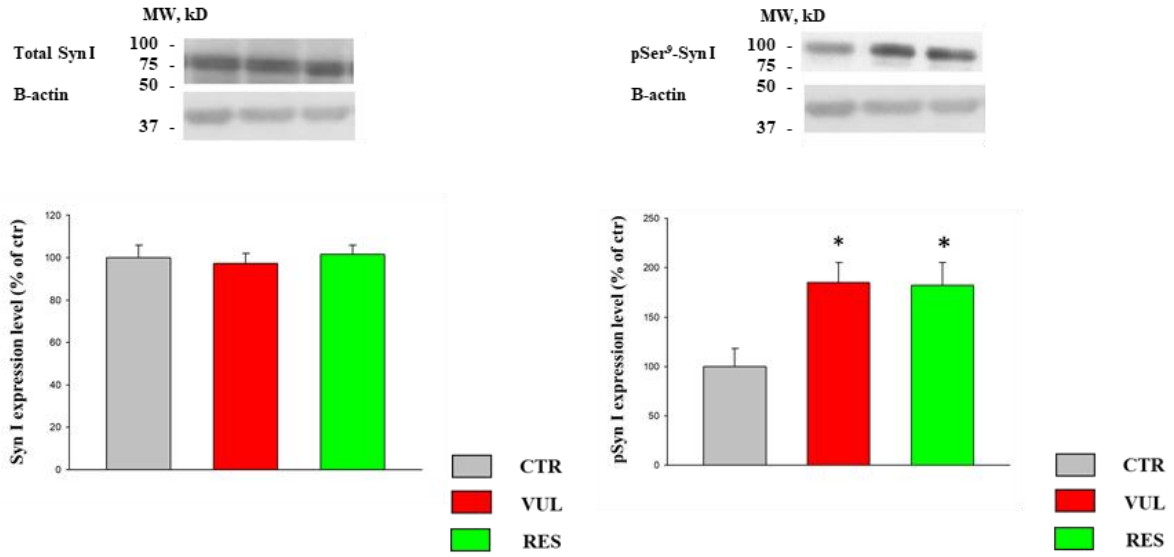

Figure 4.6.1. Total Synapsin I and pSer^{9}- Synapsin I expression in PFC synaptic membranes of VUL and RES rats at 24h after FS stress.

Expression of total Syn I (left panel) and pSer9- Syn (right panel) in PFC synaptic membranes of VUL and RES rats respect to controls at 24h after FS is reported. Proteins were determined by SDS-PAGE and Western blotting using selective antibodies (see the Methods section). Representative immunoreactive bands are reported above the respective quantification graph. Data are MEAN ± s.e.m of 10-13 independent experiments. One-way ANOVA followed by Bonferroni post hoc test. *p < 0.05 versus controls.

4.7 BASAL AND 15mM KCl EVOKED GLUTAMATE RELEASE FROM PFC GLIOSOMES 48h AFTER FS IN VUL AND RES RATS TREATED WITH KETAMINE

To verify whether an acute treatment with KET rescues the excessive stimulus-evoked Glu overflow observed in VUL rats 24 hours after FS, a set of experiments was carried out according to the experimental design illustrated in Figure 3.8. Briefly, i) rats were subjected to FS tress; ii) they were exposed to SI 24 hours later and divided in VUL and RES; iii) rats were injected with KET (i.p., 10mg/kg) or physiological solution (0.9% w/v NaCl); iv) VUL and RES rats were sacrificed for experiments 24h after KET injection (corresponding to 48h after FS stress), after having checked again their phenotype by means of SI.

The results obtained are reported in Figure 4.7 and showed that KET treatment did not modify [^{3}H]D-Asp release both under basal and depolarizing conditions, in both phenotypes. However, it is interesting to note that 48h after FS stress, the VUL vehicle-treated rats no longer showed the increase of the 15mM evoked-[^{3}H]D-Asp release observed at 24h after FS stress; thus, possibly hampering the detection of KET effect. The lack of release potentiation also indicate that the increase of [^{3}H]D-Asp release observed 24h after FS is spontaneously rescued at this time point.

These data suggest that the time point utilized to analyze the antidepressant KET effect was not the correct one. Therefore, future experiments should be planned by injecting KET 6h after FS stress and performing release experiments 18h later (24h after FS stress), when the excessive [^{3}H]D-Asp release is present.

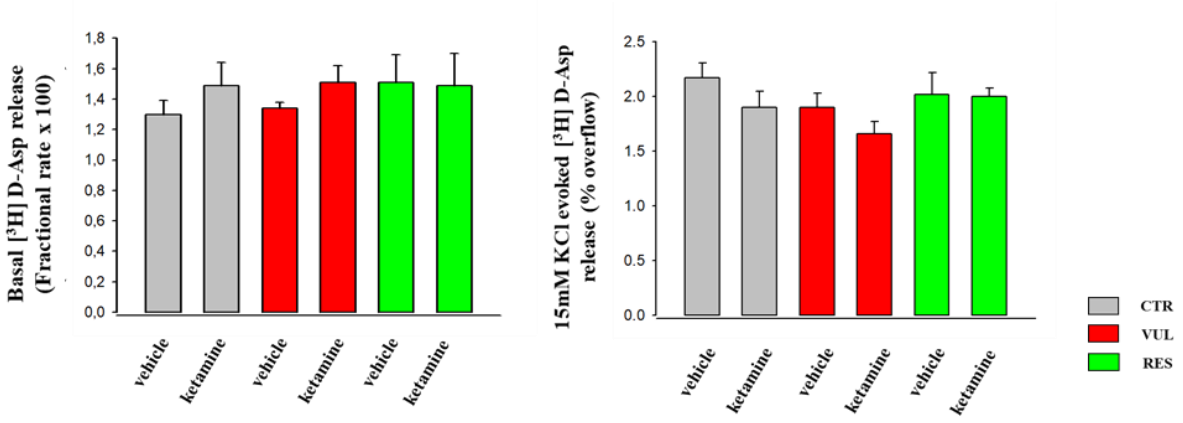

Figure 4.7. Basal (left) and 15mM evoked-[^{3}H]D-aspartate release (right) in PFC gliosomes in VUL, RES and controls animals 24h after KET injection and 48h after FS stress.

24h after the FS-stress session, animals were injected with KET (10 mg/kg, i.p.) or physiological solution (0.9% w/v NaCl) and then sacrificed 24h later. Gliosomes were prepared as described in the Methods section and exposed in superfusion to a 90s 15mM KCl depolarizing stimulus. Basal release (left panel) was expressed as the content of [^{3}H]D-Asp in the sample collected t=36-39 min of superfusion, before the introduction of the depolarizing stimulus, and are expressed as fractional rate percent. The net amount of stimulus-released neurotransmitter (right panel) was calculated by subtracting the transmitter content of the two 3-min basal release fractions (36-39 min and 45-48 min) from the release evoked in the 6-min sample collected during and after the depolarization pulse (39-45 min, stimulus-evoked release). Data are expressed as MEAN ± s.e.m. of 5-7 independent experiments. Two-way ANOVA.

4.7.1 PHENOTYPE DISTRIBUTION AFTER KETAMINE INJECTION

Since recent studies suggested that KET may exert a prophylactic action against the effects of stress over a period of 1h to 72h after administration, possibly promoting an increase in resilience (Sala et al., under revision), we assessed whether the pharmacological treatment described above could revert the VUL phenotype of the animals after FS stress; thus supporting its possible therapeutic effect, notwithstanding [^{3}H]D-Asp release that was unaffected. The anhedonic phenotype was first assessed 24h after FS stress, before injecting KET, and, in the same animals, 24h after KET administration.

Results showed that vehicle-treated VUL rats maintained their phenotype fairly constant from 24h to 48h after FS stress (Figure 4.7.1 left). Also the group of animals treated with KET did not show any rescue from the VUL phenotype (Figure 4.7.1 right). It is interesting to note a discrepancy between phenotype and functional alterations induced by stress, since ketamine, at this time, is not able to reverse from vulnerability to resilience, an indication of the establishment of maladaptive responses to stress, but this behavioral modification does not correlate with an increased Glu release.

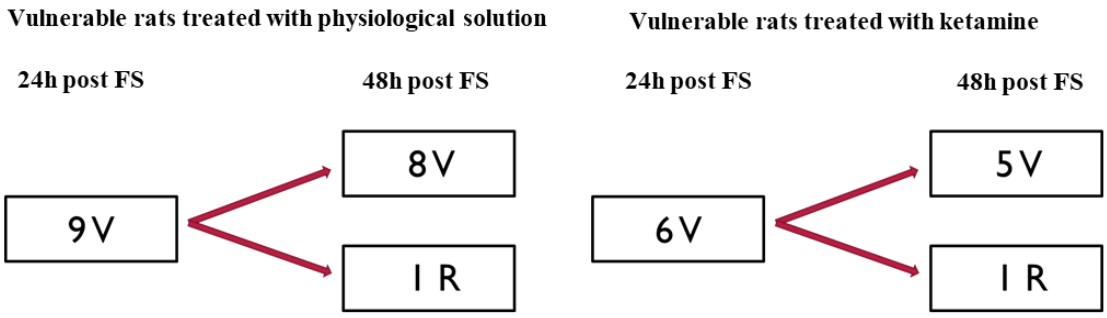

Figure 4.7.1. Phenotype distribution 24h and 48h after FS stress, corresponding to 24h after KET injection.
24h after the FS-stress session, animals were injected with KET (10 mg/kg, i.p.) or physiological solution (0.9% w/v NaCl), and then sacrificed 24h later. SI was first assessed 24h after FS, before KET administration, and then again 24h later, when sacrificed for experimental purposes. In detail, in the vehicle VUL group, only 1 rat turn to RES, spontaneously, in 24h. In the KET-treated group, only 1 KET treated VUL rat turn to RES, 24h post-KET injection.

5. DISCUSSION

Nowadays, in literature there are still a few published data on acute stress, although it is well established that this condition induces both short- and long-term consequences involving structural and functional features of the brain synapses and circuitry (Musazzi et al., 2017).

The analysis of the stress response to unveil the individual predisposition to develop a RES or VUL phenotype is still poorly investigated. The dissection of the mechanisms underlying the behavioural modifications, according to a scalar approach based on the evaluation of the molecular, synaptic and cellular changes, the circuitry modifications, the neuroarchitecture alterations is essential to characterize the pathological relevance and novel targets for the treatment (Sanacora et al., 2021). In fact, due to the biphasic nature of stress, proadaptive or maladaptive outcomes can occur depending on how the response proceeds.

The activation of the HPA axis is fundamental to face the homeostasis disturbance, through an allostatic response, and to reach a new equilibrium hence following positive, physiological, and resilient stress reaction, which allows adaptation to the changes in the surrounding environment. On the other hand, when stress is too strong or lasts for a long time, exceeding the coping capability of the individual, an allostatic load condition occurs and leads to maladaptive changes, deleterious for mental health (McEwen, 2017).

Neuropsychiatric disorders are traditionally studied in animal models through the application of protocols based on repeated or chronic stress. However, more recent evidence has highlighted that even a single stressful event is able to induce mood disorders in humans (Nestler and Hyman, 2010; Hagenaars, 2011; McEwen et al., 2014). Basing on these considerations, we administered rats with an acute stress protocol of FS, already described in the literature (Siegmund and Wotjak; 2006). The acute FS stress we used represents an important tool constituted by the combination of physical and emotional elements (Bali and Jaggy, 2015). One of the most relevant advantages is the possibility of constant monitoring over stressor intensity and duration throughout the experimental procedure (Bali and Jaggy, 2015). These features contribute defining a new reliable, time- and animal-saving model to study different pharmacologically validated models thanks to the variation of set parameters. Using this paradigm, we assessed the modifications of SI to reveal the presence of a stress-induced anhedonic phenotype. Anhedonia represents a pivotal symptom of depression and

other neuropsychiatric diseases and strictly relates to maladaptive responses (Nestler and Hyman, 2010; Christensen et al., 2011; Franklin et al., 2012).

VUL animals showed a significant reduction of SI compared to control and resilient rats at each time point analyzed after FS stress (6, 24 and 48h), whereas the RES group did not modify sucrose assumption habits compared to control; even, they showed an increase in the consumption of the sucrose solution 24h after FS stress. This trend reflects long-lasting alterations induced by acute stress in the behavioural component.

Observing the distribution of VUL and RES rats at the same time points after FS stress, it emerged that most of the population analyzed at 6h was susceptible to the stressful event (about 70% of the animals), with a reduction of this number at 24 hours; thus supporting the fact that part of vulnerable rats spontaneously modifies its phenotype, moving from a maladaptive condition to an adaptive one. Unexpectedly, this percentage rise again 48 hours after stress. This last datum is in contrast with the expectation that the percentage of vulnerable rats should be constant from 24 to 48h after FS stress or even further decrease. The reported occurrence may be due to the low number of animals analyzed at 48h after the stressful event.

Data obtained in animals in our experiments seem opposite to the stress response in humans, where less than 20% of individuals exposed to a stressful situation develops stress-related disorders, whereas the remaining population successfully adapt to significant stressors (Han and Nestler, 2017). The trend observed in rats is probably due to the limited sample numerosity and therefore it might not be representative of what occurs on a large scale in humans.

One aspect that deserves attention concerns the onset of anhedonia already 6h after the acute stress event, thus validating this model for the identification of behavioural changes related to vulnerability or resilience. This feature open to the possibility of early identifying subjects predisposed to maladaptive responses, with the consequent advantage of defining an effective pharmacological window to act promptly in the management of the possible damage triggered by the acute stress event.

Then, one of our priorities was to investigate the consequences of the stressful event on Glu transmission, since this amino acid represents the major excitatory transmitter involved in the structural and functional changes in the brain related to mood disorders (McEwen, 1999). Therefore, we assessed whether the different phenotypes could differently

associate to modifications of Glu release, focussing, in this thesis, mainly on astrocytes, since the discoveries of the last twenty years have revealed an active role of these cells in the CNS also at the synaptic level. The initial knowledge, considering astrocytes as unexcitable cells has given way to a more complex scenario where they exert cell-to-cell signalling, receiving incoming stimuli from neurons, due to the presence of membrane receptors, and synthesizing and releasing gliotransmitters, which in turn regulate the activity of the neuronal counterpart (Harada et al., 2016).

To study neuronal presynaptic and astrocyte peri synaptic Glu release we utilized synaptosomes or gliosomes in superfusion, according to a technique set up in our laboratory and considered the method of choice for this type of studies (Raiteri and Raiteri 2000).

Synaptosomes are isolated nerve endings obtained by CNS homogenization and purification by a discontinuous Percoll gradient, which retain most of the in vivo features of presynaptic terminals, such as the presence of functional ionic channel expression, providing the proper neuronal membrane potential, the maintenance of the machinery essential for neurotransmitters synthesis and release, functional mitochondria, providing local energy for the metabolic needs, expression of release-regulating receptors and transporters (Bonanno and Raiteri, 1994),

Gliosomes, that are purified from synaptosomes during gradient centrifugation (Nakamura et al., 1993; Stigliani et al.2006), are representative of the astrocytic areas around the synapses and thanks to their peculiar purification method they do not express other glial (microglial, oligodendroglial) nor neuronal markers (Stigliani et al., 2006; Karney et al., 2014). They represent the experimental model more reliable for the study of glio-transmitters release mechanisms, since they maintain a major part of functional properties belonging to in situ astrocytes, that allow them to be in turn able to synthesize, store, release and re-uptake neurotransmitters (Stigliani et al., 2007; Paluzzi et al., 2007).

According to the above-mentioned superfusion technique, synaptosomes or gliosomes were layered on large microporous filters (diameter 2.5 cm) in a small amount (<100 µg protein/filter) that will constitute less than a monolayer and up-down superfused. Many studies have demonstrated that, under these conditions, possible indirect effects mediated by substances released by neighbouring particles are minimized. Therefore, the changes of Glu release here measured are unequivocally due to features confined to Glu-releasing synaptosomes or gliosomes and, consequently, the biochemical modifications can be likely ascribed to specific phenomena taking place into these particles, without external

interference. Of course, these advantages are balanced by the restraint that this experimental paradigm does not account for possible modifications due to the anatomical and functional connections, that occur *in vivo*.

Basal release represents an index of resting Glu levels (Fatt and Katz, 1952). This component is important for the synaptic networks, involving their maturation and stability, regulation of spike timing, maintenance of synaptic strength and regulation of responsiveness at the postsynaptic level, thus playing a role in homeostatic synaptic plasticity (Kavalali et al., 2006; Vyleta et al., 2011; Kononenko and Haucke, 2012). We observed here an augmented rate of the basal Glu release from PFC synaptosomes of vulnerable rats 24h after the stressful event, Previous work also observed a rapid enhancement of the stimulus-evoked Glu release from synaptosomes freshly purified from PFC immediately after the completion of the acute FS stress protocol (Treccani et al., 2014; Popoli et al., 2012; Whittaker., 1993; Raiteri, 1983; Ghijsen, 2003).

In addition, a correlation was identified between the rapid strengthening of glutamatergic transmission and the activation of hypothalamic-pituitary-adrenal axis (HPA), leading to an increase of corticosterone (CORT) levels in FS stress-exposed rats (Treccani el al., 2014). Interestingly, CORT and stimulus-evoked Glu release showed a different trend at different times after the FS stress event. CORT instantly reached the concentration peak, then normalized in 2 hours, whereas the excitatory neurotransmitter release was detectable at 24h after FS stress, only (Musazzi et al., 2017). Interestingly, an inverse correlation was observed between CORT serum levels and Glu release in the resilient phenotype, supporting the hypothesis of a differential mechanism at the basis of the capability of the two phenotypes to face stress exposure.

In light of these evidence in synaptosomes, Glu release from gliosomes at different time points (0h, 6h, 24h and 48h) after FS stress was assessed. In fact, several papers have pointed out that, although the effects of acute and chronic stress have always been considered not superimposable, the consequences of these two distinct protocols do not exert different outcomes since also a strong single stressful event can have long-term consequences (Musazzi et al., 2017).

Immediately after the single stressful event, as well as 6h later, a significative increase of evoked Glu release was observed in synaptosomes obtained from stressed rats, as previously demonstrated (Musazzi et al 2010; Musazzi et al., 2017), whereas no differences

were detected in gliosomes. Moreover, 24h after the stressful event, an increase of Glu basal release was detected in synaptosomes of vulnerable rats, whereas a significant enhancement of the stimulus-evoked Glu release was present in both VUL and RES rats. At this same time, gliosomes did not show any increase of basal release but of the stimulus-evoked Glu release only in the vulnerable population. In PFC gliosomes, the analysis of Glu release 48h after FS stress did not highlight release differences neither in the vulnerable nor in the resilient rats, when compared to non-stressed rats.

This time course points out that the stress response in the neuronal population is immediately triggered and present in both phenotypes, whereas that of the astrocytic component requires longer time to activate the plastic modifications necessary to detect significant release changes, which are expressed in the form of selective increase of Glu release in VUL rats. The stimulus-evoked Glu release in gliosomes from vulnerable animals normalized 48h after FS stress, indicating that the alterations last no more than 24h, time window after which a rebalancing of the system could be observed.

On the basis of these data, it could be hypothesized that, in neurons from VUL rats, the onset of the maladaptive response is attributable to the fact that the mechanisms responsible cannot correctly re-establish Glu homeostasis. This occurrence probably bases on two main reasons: i) an inappropriate control of the response timing and/or ii) a hyperactivation of the inhibitory response, due to the observed enhancement of the spontaneous release of Glu.

Another aspect that we investigated was the assessment of the mechanisms underlying the excessive stimulus-evoked Glu release 24h after FS stress in PFC gliosomes prepared from VUL rats. Results pointed out that the presence of the Glu transporter inhibitor TFB-TBOA in the superfusion medium significantly reduced the 15 mM KCl-evoked Glu release, supporting the idea that transporters play a role in the observed neurotransmitter dysregulation, sustaining the release of Glu when working in the reverse mode, due the Na^+ imbalance driven by KCl depolarization. On the other hand, the study of the mechanisms at the basis of the increased Glu release from PFC synaptosomes 24h after FS stress showed that the total expression of Synapsin was unmodified, while the phosphorylation levels at Ser^9 site, a fundamental requirement to trigger the rapid increase of Glu release induced by stress (Treccani et al., 2014), was significantly increased. This evidence, along with the calcium dependence of the stimulus-evoked release of Glu, play in favour of a plastic change at the exocytotic mechanism level.

As previously reported, a role of glucocorticoid and mineralcorticoid receptors was demonstrated to support the excessive Glu release from PFC synaptosomes in FS rats (Treccani et al., 2014). The receptor expression was evaluated in the synaptosomal nuclear and membrane fractions in the RES and VUL phenotypes. No differences were detected in the receptor expressions and activation in the membrane fraction. MR_s expression was increased in RES animals 24h after FS stress in the nuclear component. Conversely, GR_s were more expressed in VUL rats. These data are in line with the hypothesis that the increase of Glu release is also mediated by mechanisms involving the genomic component and acting in a delayed manner (Treccani et al., 2014). In this context, it is plausible to assume that RES and VUL animals adopt different mechanisms to face acute stress by exploiting the action of GR_s and MR_s as nuclear transcription factors.

Finally, when studying the effects of the pharmacological treatment with KET, we observed that this molecule did not reverse the vulnerable phenotype, thus supporting the absence of a fast antidepressant action. Our decision to investigate the possible rescue of the acute stress effects by this molecule derived from literature evidence reporting that chronic treatment with conventional antidepressants, as well as an acute treatment with KET, reversed the anhedonic behaviour induced by a chronic stress protocol (Willner et al. 1987; Papp et al. 1996; Larsen et al., 2010; Zhang et al., 2010; Li et al., 2011; Choi et al., 2015; Sun et al., 2016; Hare et al., 2017; Papp. et al., 2017).

We have previously demonstrated that ketamine, acutely administered i.p. (10mg/kg) 24h and 72h before FS stress, normalized the excessive Glu release from PFC synaptosomes (Sala. et al., 2021 submitted), thus showing a preventive role in blocking the maladaptive response. Moreover, in the same paper, we have also demonstrated that KET reduced the excessive Glu release 24h after FS stress when administered 6h after the stressful event, thus supporting its therapeutic role. Nevertheless, it is important to highlight that in this study, the KET administration effects were assessed without differentiate between VUL or RES rats.

Here, we analyzed the effect of KET on the excessive Glu release observed in gliosomes, as a neuronal counterpart. For this purpose, we administered KET 24h after FS stress, the time point where the excessive Glu release was evidenced, distinguishing the VUL or RES phenotype, and we measured its effect on Glu release 24h later, corresponding to 48h after FS stress. The lack of beneficial effects we observed is probably due to the timing chosen for the study. In fact, 48h after FS stress, Glu release was spontaneously normalized in VUL rats respect to the controls, thus probably preventing the detection of any beneficial

effect of the drug. A similar trend was also observed in the KET-induced restoring of the anhedonic phenotype. For this reason, in future studies, experiments aimed at identifying a more appropriate time point should be planned.

The present data show that neurons and astrocytes differently contribute to the plastic changes of the synapse strength after acute stress, acting at the release of Glu and contributing to define the RES or the VUL phenotype in rats. These differences are both biochemical and temporal, suggesting that the most precocious modifications take place at the presynaptic level and then sprout to the peri synaptic astrocyte areas, thus possibly reinforcing the neuronal alterations.

6. CONCLUSIONS

Although chronic stress protocols are used in most cases to produce animal models for the investigation of neuropsychiatric diseases, they are able to reproduce only the endpoint of several adaptive modifications taking place in the brain and the body during the stress response (Musazzi et al., 2017). Data show, instead, that also a single stressful event, as well as the exposure to repeated stressors, can trigger both rapid and long-lasting functional and molecular changes of excitatory synapses in PFC.

In our study, we used a well-established acute stress protocol with an innovative purpose, the identification of the VUL/RES phenotype in order to dissect the short- and long-term consequences for the identification of the factors that shift the physiological stress response into maladaptive alterations. In particular, little is known about the rapid changes induced by stress at the synaptic level, probably mediated by non-genomic mechanisms and linked to local (synaptic) processes (Karst et al., 2005; Joëls et al., 2012). The alterations of the glutamatergic system, the main consequence of stress in the hippocampal and cortical areas, is considered a main feature of neuropsychiatric pathophysiology (Duman and Aghajanian, 2012; Lener et al., 2017; Murrough et al., 2017; Musazzi et al., 2017, 2010; Sanacora et al., 2012). Convergent evidences have shown that several stressors acutely increase Glu efflux into cortical and limbic areas, including HPC, AG and PFC, and that this effect is at least in part fostered by CORT (Musazzi et al., 2010; Sanacora et al., 2012; Bagley e Moghaddam, 1997; Lowy et al., 1993; Reznikov et al., 2007; Venero and Borrell, 1999), which certainly takes part in Glu release modification mediated by FS-stress.

It can be concluded that both neurons and astrocytes play an important role in acute stress-induced Glu excitotoxicity, although neuronal Glu dysfunction occurs immediately after the stressful event, whereas longer time is required for its establishment in the astrocytic component. Moreover, some molecular and functional alterations are common in both RES and VUL animals, while others are specific hallmarks of a unique phenotype and characterized by a peculiar temporal onset. Disclosing the multiple biological factors and their interactions, resulting in vulnerability or resilience of the stress response, is fundamental for the understanding of stress-related disorders and the characterization of new druggable targets.

As regards KET, in light of the now consolidated data showing that this drug can exert a prophylactic action against the effects of a single stressful event, further experiments are needed

to assess its potential therapeutic action in counteracting already established stress-induced alterations, with the aim of introducing innovative pharmacological strategies employing more efficient antidepressant drugs.

7. PUBLICATIONS

- **Acute ketamine facilitates fear memory extinction in a rat model of PTSD along with restoring glutamatergic alterations and dendritic atrophy in the prefrontal cortex (accepted for publication).**

  Sala N., Paoli C., Bonifacino T., Mingardi J., Schiavon E., La Via L., Milanese M., Tornese P., Datusalia A.K., Rosa J., Facchinetti R., **Frumento G.**, Carini G., Salerno Scarzella F., Forti L., Barbon A., Bonanno G., Popoli M. and Musazzi L. Frontiers in Pharmacology.

- **Nearly 30 Years of Animal Models to Study Amyotrophic Lateral Sclerosis: A Historical Overview and Future Perspectives (2021).**

  Bonifacino T., Zerbo A.R., Balbi M., Torazza C., **Frumento G.**, Fedele E., Bonanno G. and Milanese M. Int. J. Mol. Sci.; 22: 12236. doi: 10.3390/ijms222212236.

- **Blocking glutamate $mGlu_5$ receptors with the negative allosteric modulator CTEP improves disease course in $SOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis (2021).**

  Milanese M., Bonifacino T., Torazza C., Provenzano F., Kumar M., Ravera S., Zerbo A.R., **Frumento G**, Balbi M., Nguyen T.P.N., Bertola N., Ferrando S., Viale M., Profumo A., Bonanno G. Br J Pharmacol.; 178(18): 3747-3764. doi: 10.1111/bph.15515.

- **A2A-D2 Heteromers on Striatal Astrocytes: Biochemical and Biophysical Evidence (2019).**

  Pelassa S., Guidolin D., Venturini A., Averna M., **Frumento G.**, Campanini L., Bernardi R., Cortelli P., Buonaura G.C., Maura G., Agnati L.F., Cervetto C., Marcoli M. Int J Mol Sci.; 20(10):2457. doi: 10.3390/ijms20102457.

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