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ACUTE KETAMINE RESCUES CELLULAR AND MOLECULAR CHANGES INDUCED BY CHRONIC MILD STRESS IN VULNERABLE RATS

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ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methyl-4-
isoxazolepropionic acid

BDNF: brain-derived neurotrophic factor

BDNF-1: BDNF transcript containing exon 1

BDNF-2: BDNF transcript containing exon 2

BDNF-4: BDNF transcript containing exon 4

BDNF-6: BDNF transcript containing exon 6

CaMKII: Ca^{2+}/calmodulin-dependent kinase II

CNT: control

CMS: chronic mild stress

CMS-R: chronic mild stress-resilient

CMS-V: chronic mild stress-vulnerable

CMS-V+KET: chronic mild stress-vulnerable
treated with ketamine

CNS: central nervous system

CORT: corticosterone

DMS-5: Diagnostic and Statistical Manual of
Mental Disorders 5

eEF2: eukaryotic elongation factor 2

eEF2K: eukaryotic elongation factor 2 kinase

GABA: gamma-aminobutyric acid

GBD: Global Burden of Disease

GR: glucocorticoid receptor

H: homogenate

HB: homogenization buffer

HPA: hypothalamic–pituitary–adrenal

HPC: hippocampus

KET: ketamine

LB: lysis buffer

LP1: synaptic membranes

MDD: major depressive disorder

MR: mineralocorticoid receptor

mGluR2: metabotropic glutamate receptor 2

mTOR: mammalian target of rapamycin

NMDA: N-methyl-D-aspartate

o/n: overnight

P2p: synaptosomes

PB: physiological buffer

PhI: phosphatase inhibitors

PI: protease inhibitors

PFC/FC: prefrontal/frontal cortex

qPCR: quantitative Real-Time PCR

RT: room temperature

Ser: serine

SPT: sucrose preference test

Thr: threonine

VEH: vehicle

ABSTRACT

Increasing evidence has associated dysfunction of the glutamate system with the pathophysiology of stress-related neuropsychiatric disorders, including major depressive disorder. Clinical studies on depressed patients have shown consistent volumetric and functional changes in brain areas where glutamatergic transmission is predominant, such as hippocampus (HPC) and prefrontal and frontal cortex (PFC/FC). In parallel, preclinical studies on stress-based animal models of depression reported impaired glutamate neurotransmission and dendritic arborisation in the same brain regions affected in patients.

Intriguingly, consistent evidence reported that a single sub-anaesthetic dose of the NMDA receptor antagonist ketamine (KET) induces rapid and sustained antidepressant effect, both in patients and in rodent models of depression. However, molecular mechanisms underlying KET therapeutic effect remain poorly understood.

Using a chronic mild stress (CMS) rat model of depression, we aimed at studying the functional, morphological and molecular changes associated with fast antidepressant action of KET.

Rats were subjected to CMS for 5 weeks. Sucrose preference test allowed to distinguish stress resilient (CMS-R) from vulnerable (CMS-V) rats. 10 mg/kg KET, acutely administered to CMS-V 24 hours before sacrifice, reversed anhedonic behavior. CMS induced significant phenotypic changes in all CMS rats, although the decrease in body weight gain and the increase of circulating corticosterone levels and adrenal glands weight were higher in CMS-V.

A decrease in basal and depolarization-evoked glutamate release was selectively measured in HPC synaptosomes in superfusion from CMS-V, and in PFC/FC synaptosomes from CMS-R. Intriguingly, KET restored basal, but not depolarization-evoked, glutamate release in CMS-V.

Area- and subcellular fraction-specific modifications in the expression and phosphorylation levels of selected proteins involved in glutamate release and stress response were measured by Western Blot in total homogenate, synaptosomes and synaptic membranes from HPC and PFC/FC.

qPCR revealed a significant reduction in total-BDNF and BDNF splice variants transcripts in HPC and PFC/FC of all CMS rats, including those treated with KET. However, in situ hybridization studies found reduced dendritic trafficking of total-BDNF, and BDNF-2/BDNF-6 variants mRNAs in CA1 and CA3 of CMS-V, while KET treatment, although not reversing changes in BDNF mRNA levels, completely rescued dendritic trafficking in CA3 of CMS-V. Morphological analysis of CA3 pyramidal neurons in Golgi-Cox stained sections showed a reduction in total length and branching of apical, but not basal, dendrites. KET restored these changes to control levels.

Overall, our results show that chronic exposure to mild stress induces functional and morphological alterations in HPC and PFC/FC of vulnerable rats. Interestingly, a single administration of KET was able to reverse most of these deficits.

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ABSTRACT (Italian)

Numerosi studi hanno associato la disfunzione del sistema glutammatergico con la fisiopatologia dei disturbi neuropsichiatrici correlati allo stress, compreso il disturbo depressivo maggiore. Studi clinici su pazienti depressi hanno mostrato variazioni volumetriche e funzionali in aree cerebrali dove prevale la trasmissione glutammatergica, come l'ippocampo (HPC) e la corteccia prefrontale e frontale (PFC/FC). In parallelo, studi preclinici su modelli animali di depressione basati sullo stress hanno osservato alterazioni della neurotrasmissione glutammatergica e dell'arborizzazione dendritica nelle stesse aree cerebrali coinvolte nei pazienti.

Studi recenti hanno mostrato che una singola dose sub-anestetica di ketamina (KET), un antagonista del recettore NMDA, induce un rapido e sostenuto effetto antidepressivo, sia nei pazienti depressi, che nei modelli animali di depressione. Tuttavia, i meccanismi molecolari che sottendono l'effetto terapeutico di KET rimangono al momento poco conosciuti.

Utilizzando il chronic mild stress (CMS), un modello animale di depressione, abbiamo studiato i cambiamenti funzionali, morfologici e molecolari associati all'azione antidepressiva rapida di KET. I ratti sono stati sottoposti a CMS per 5 settimane. Attraverso il test di preferenza per il saccarosio siamo riusciti a distinguere i ratti resilienti (CMS-R) da quelli vulnerabili (CMS-V) allo stress. KET (10 mg/kg), somministrata in acuto ai CMS-V 24 ore prima del sacrificio, è stata in grado di invertire il comportamento anedonico. Il CMS ha indotto importanti cambiamenti fenotipici in tutti i ratti stressati, anche se la diminuzione nell'incremento ponderale, l'aumento dei livelli serici di corticosterone e l'aumento del peso delle ghiandole surrenali sono risultati più elevati nei CMS-V.

Una riduzione del rilascio di glutammato, sia basale, che evocato da depolarizzazione, è stata osservata selettivamente in sinaptosomi in superfusione da HPC di CMS-V e nei sinaptosomi da PFC/FC di CMS-R. KET è stata in grado di ripristinare il rilascio di glutammato basale, ma non quello depolarizzato, nei CMS-V.

Mediante Western Blot, sono state misurate alterazioni area e frazione cellulare specifiche nei livelli di espressione e di fosforilazione di determinate proteine coinvolte nel rilascio di glutammato e nella risposta dello stress, in omogenato totale, sinaptosomi e membrane sinaptiche da HPC e PFC/FC.

Tramite qPCR abbiamo rivelato una significativa riduzione dei trascritti totali di BDNF e delle sue varianti di splicing in HPC e PFC/FC di tutti i ratti stressati, inclusi quelli trattati con KET. Tuttavia, studi di ibridazione *in situ* hanno riscontrato un ridotto traffico dendritico di mRNA totale di BDNF e BDNF-2/BDNF-6 in CA1 e CA3 di CMS-V, mentre il trattamento con KET, pur non ripristinando i cambiamenti nei livelli di mRNA totale di BDNF, è stato in grado di ristabilire il traffico dendritico in CA3 di CMS-V. L'analisi morfologica dei neuroni piramidali in CA3, in sezioni marcate con il metodo di Golgi-Cox, ha mostrato una riduzione della lunghezza totale e della ramificazione dei dendriti apicali, ma non di quelli basali. KET ha ripristinato tali modifiche ai livelli di controllo.

Nel complesso, i nostri risultati mostrano che l'esposizione allo stress cronico induce alterazioni funzionali e morfologiche in HPC e PFC/FC di ratti vulnerabili. È interessante notare che una singola amministrazione di KET è in grado di recuperare la maggior parte di questi deficit.

1. INTRODUCTION

1.1 Mental health and mood disorders

The World Health Organization defines mental health as *"a state of well-being in which the individual realizes his or her own abilities, can cope with the normal stresses of life, can work productively and fruitfully, and is able to make a contribution to his or her community"* (*World Health Organization, 2013*). A critical implication of this definition is that the maintenance and restoration of mental health should be regarded as a fundamental concern for public health (*Prince et al., 2007; Galderisi et al., 2015)*.

Nevertheless, up to 30% of the population worldwide has some form of mental disorder that affects its thoughts, perceptions, emotions and behavior, and almost two-thirds of those people do not receive adequate treatments (*Kohn et al., 2004; Ngui et al., 2010)*.

Mood disorders are a group of psychiatric conditions, which profoundly alter the mood and feelings of affected people, representing the most frequently diagnosed mental illnesses. Among these, major depressive disorder is the most prevalent mood disorder in the world population (*GBD Disease and Injury Incidence and Prevalence Collaborators, 2016*).

1.2 Major depressive disorder

Major depressive disorder (MDD), also commonly called major depression, unipolar depression or clinical depression, is a chronic and debilitating mental disorder that strongly impairs an individual's ability to function and cope with daily life (*Fava and Kendler, 2000; Willner et al., 2013; Otte et al., 2016*). The fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) identifies MDD as the most severe

among the depressive disorders, a class of pathologies which share the presence of low or irritable mood, together with significant somatic and cognitive changes (*American Psychiatric Association, 2013*).

1.2.1 Epidemiology

The latest estimates of the Global Burden of Disease (GBD) epidemiological study, made available by the World Health Organization in 2016, indicate that MDD affects the health of approximately 322 millions of people, corresponding to 4.4% of the world population (*GBD Disease and Injury Incidence and Prevalence Collaborators, 2016*). Depression is more common among females (5.1%) than males (3.6%), and its prevalence is higher in older adulthood (above 7.5% in females and 5.5% in males with age between 55 and 74 years) (*Fig. 1.1*).

Figure 1.1: Prevalence of major depressive disorder in the world population

Global prevalence (% of the world population) of MDD **(a)** by sex and world region, and **(b)** by sex and age. Data from the 2015 Global Burden of Disease study, promoted by the World Health Organization.

MDD was ranked the third leading cause of disability worldwide in 2015 (*GBD Disease and Injury Incidence and Prevalence Collaborators, 2016*), and projections made by the World Health Organization suggest that it will reach the first place by 2030 (*World Health Organization, 2013*).

Depressed individuals also have shorter life expectancy, being more susceptible to other medical conditions, like heart disease, and being more prone to high-risk behaviors, including drug and alcohol abuse (*Ferrari et al., 2013; Walker et al., 2015*). Moreover, nearly 60% of the people died by suicide (approximately 800000 individuals each year) had a history of clinical depression, making MDD the major contributor to suicide (*GBD Disease and Injury Incidence and Prevalence Collaborators, 2016*).

Major depression also represents a relevant economic burden for society. Taking in consideration medical services, pharmaceutical costs, reduced work productivity, unemployment and suicide-related expenses, MDD was estimated to cost, only for the United States, more than 200 billions of dollars each year (*Birnbaum et al., 2010; Greenberg et al., 2015*).

1.2.2 Symptoms

MDD is a clinically complex and heterogeneous pathology, characterized by the chronic presence of different mental and physical alterations. Two of these alterations are generally acknowledged as core symptoms of MDD: depressed mood and loss of interest or pleasure (*Fava and Kendler, 2000; Wong and Licinio, 2001; Hasler et al., 2004; Kennedy, 2008*).

Low mood affects depressed people in a different way from the ordinary feeling of sadness or moodiness that anyone can experience in reaction to adverse life events. Clinically diagnosable depressed mood pervades every aspect of the life of affected individuals, substantially impairing their behavior, social relationships, occupational life and sense of well-being (*Otte et al., 2016; American Psychiatric Association, 2013*).

Depressed people also exhibit a marked decrease in interest in many, or nearly all, the activities that they formerly considered pleasurable (*Hasler et al., 2004*). This phenomenon, defined "anhedonia", is a specific feature of depressed patients (*Fawcett et al., 1983*) and is supposedly linked to a dysfunction of the brain reward system (*Nestler et al., 2002; Pizzigalli, 2014*). The lack of reactivity to pleasurable stimuli results in

extremely impaired behavior, including loss of motivation, withdrawal from social situations and reduced sex drive (*American Psychiatric Association, 2013*).

According to DSM-5 diagnostic criteria, MDD may be recognized by the concomitant presence, for a time period of at least two weeks, of one or both core symptoms of depression, together with four (or more) of the following symptoms:

- altered appetite, resulting in significant weight loss or gain;
- altered sleep, including insomnia, early-morning waking or hypersomnia;
- psychomotor agitation, including anxiety, irritability or rumination of thoughts;
- cognitive dysfunctions, including diminished ability to think, concentrate, remember or take decisions;
- physical dysfunctions, including fatigue, loss of energy and pain;
- negative thoughts, including feelings of hopelessness, helplessness, worthlessness, excessive guilt or self-hatred;
- recurrent thoughts of death and suicidal ideation.

1.2.3 Diagnosis

The diagnosis of MDD is generally conducted by a psychiatrist who records the patient's symptoms and biographical history, in order to identify relevant biological, psychological and social factors that may be impacting on its mood (*Otte et al, 2016*). The mental health examination often includes the use of rating scales, such as the Hamilton Rating Scale for Depression (HAM-D) and the Montgomery-Asberg Depression Rating Scale (MADRS), or clinical interviews, such as the Structured Clinical Interview for DSM (SCID) (*Kennedy, 2008; Pettersson et al., 2015*). Despite these evaluations, it is estimated that only 50% of depression cases are correctly recognized (*Goldman et al., 1999; Bech, 2006*).

A growing field of research has developed novel tools to provide more accurate diagnosis for depression in the last years; however, no clinically relevant biomarkers have yet been found (*Gadad et al., 2017; Strawbridge et al., 2017*). Moreover, the lack of economic resources and trained health-care providers (*World Health Organization, 2013*), together with the social stigma associated with mental disorders (*Corrigan and Watson, 2002*), often lead to misinterpreted depressive symptoms, delayed diagnosis and inappropriate pharmacological treatments, further worsening the burden of MDD (*Seelig and Katon, 2008*).

1.3 Risk factors of major depressive disorder

Due to its clinical heterogeneity, it has been difficult to elucidate the neurobiological underpinnings of MDD. The most accredited hypothesis indicates that depression may be the outcome of an intricate combination of genetic and environmental factors, that predispose susceptible individuals to develop MDD (*Fava and Kendler, 2000; Jaffee and Price, 2007; Uher, 2008; Schmitt et al., 2014; Uher, 2014a*) (*Fig. 1.2*).
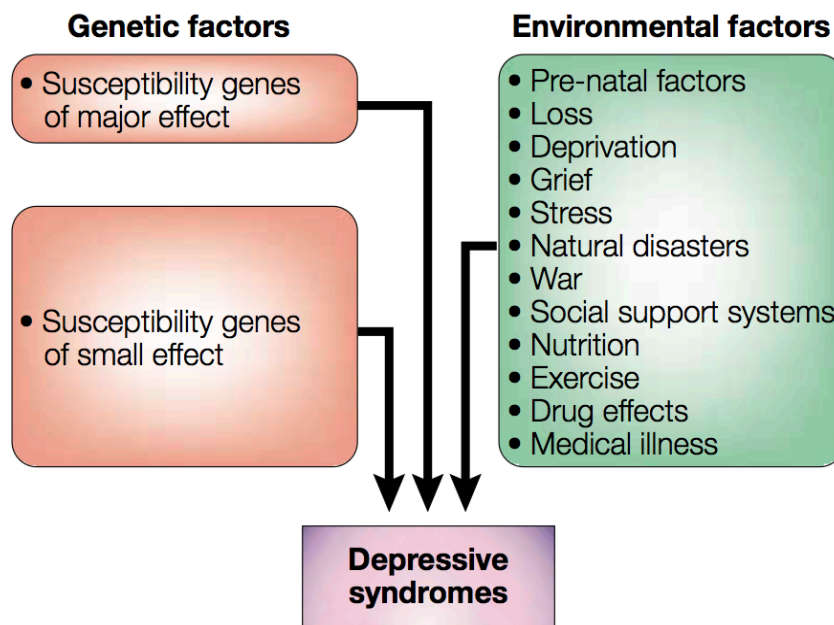
Figure 1.2: Gene-Environment interaction in the onset of MDD

The interactions between genetic predisposition and different environmental factors lead to the development of depressive syndromes. From *Wong and Licinio, 2001.*

1.3.1 Genetic risk factors

About 40% of the risk of MDD is estimated to be related to genetics, making depression a highly heritable disorder (*Fava and Kendler, 2000; Flint and Kendler 2014*). For this reason, several genetic studies tried to identify genes specifically involved in the development of MDD (*López-León et al., 2008; Psychiatric Genetic Consortium, 2013*). However, due to the complexity of major depression and to the high number of genes possibly involved, the search for candidate genes has not provided relevant results up to date (*López-León et al., 2008; Flint and Kendler 2014*). However, different specific

genetic polymorphisms that may directly interact with environmental factors in the onset of MDD have been identified (*Uher, 2014b*).

The first evidence of this interplay came from the work of Caspi and collaborators, who reported that a common length variant in the 5-HTTLPR region of the gene coding for the serotonin transporter (SLC6A4) increased the risk of depression in people with a maltreatment history (*Caspi et al., 2003*). 5-HTTLPR was also found to interact with the Val66Met single nucleotide polymorphism of the brain-derived neurotrophic factor (BDNF) gene in predicting depression in children subjected to maltreatment (*Kaufman et al., 2006*). Furthermore, the Val66Met polymorphism of BDNF has been repeatedly reported to interact with stressful life events in the development of MDD, with Met allele carriers being more at risk of depression (*Aguilera et al., 2009; Notaras et al., 2015*).

In line with these observations, other studies in the last years have identified possible interactions between genetic variants and environmental factors in the onset of MDD, such as the variable number tandem repeats of the Monoamine oxidase A gene (MAOA) (*Chicchetti 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., 2009*), or the 22/23EK and 9beta polymorphisms of the Glucocorticoid Receptor gene (NR3C1) (*Bet et al., 2009*).

Intriguingly, epigenetic mechanisms, such as chromatin remodeling, histone post-translational modifications, DNA methylation and non-coding RNAs, have also been shown to mediate the effects of environmental factors on gene transcription, both at critical stages of life and across different generations (*Provençal and Binder, 2015; Denhardt, 2017*).

1.3.2 Environmental risk factors: the role of stress

Vulnerability to depression is only partly genetic; multiple biological and psychological factors may affect the health and mood of a person at any point of his life (*Schmitt et al., 2014*) (*Fig. 1.2*).

Depression may be the result of particular medications (such as anticonvulsants, antipsychotics, cardiac drugs, interferon therapy, or hormonal agents), or of substance abuse and withdrawal (as in the case of alcohol, sedatives, opioids, stimulants and hallucinogens) (*Brook et al., 2002; Rogers and Pies, 2008; American Psychiatric*

Association, 2013). Depression may also be comorbid with other chronic diseases (such as cardiovascular illnesses, Parkinson's disease, cancer, chronic pain, or multiple sclerosis), or with other psychiatric conditions (like post-traumatic stress disorder, anxiety, schizophrenia or attention deficit hyperactivity disorder) (*Rickards, 2005; American Psychiatric Association, 2013*).

Psychological factors however seem to be the main contributors to the environmental risk of MDD (*Schmitt et al., 2014;*). Stress is commonly acknowledged as the major predisposing and triggering factor for depression, and MDD itself is considered the most common stress-related disorder (*Kessler, 1997; Nestler et al. 2002; Hammen, 2005; McEwen, 2013; Popoli et al., 2014; Uher, 2014b*). Emotional trauma (including grief, social rejection, physical or sexual abuse), major life changes (such as bereavement, health condition, childbirth, financial difficulties, natural disasters, war) and adversities in childhood or adolescence (including maltreatment, neglect or bullying) are widely recognized as important risks for mental health (*Heim et al., 2007; Meltzer-Brody et al, 2011; American Psychiatric Association, 2013; Lindert et al., 2014; Uher, 2014a*). Moreover, studies in community samples showed that up to 80% of major depressive episodes are preceded by significant stressful events (*Hammen, 2005; Pizzigalli, 2014*).

1.3.3 The stress response

Despite being a well-recognized and almost universally experienced phenomenon, the notion of stress was not conceptualized until the last century. Hans Selye, the pioneer of the research field on stress, defined stress as a "*non-specific response of the body to any demand placed upon it*" (*Selye, 1956*). Since then, the word "stress" has been used to indicate the biological and psychological processes activated in reaction to those challenges (or "stressors"), able to alter the physiological homeostasis of the organism (*Joëls and Baram, 2009; McEwen, 2013; Popoli et al., 2012*).

Stress is a positive and essential evolutionary response that allows individuals to cope with harmful stimuli and adapt to the changing environment (*Gold et al., 2005; Joëls and Baram, 2009*). In a physiological context, when a stimulus is perceived as a potential or actual threat, specific brain regions orchestrate a sequence of hormonal, biochemical, functional and behavioral adjustments (*Selye, 1936*), aimed at reestablishing the disrupted homeostasis, a process known as "allostasis" (*McEwen and Stellar, 1993*) (*Fig. 1.3*).

Usually, these changes rapidly return to baseline levels after stress cessation. However, when stress is overwhelming or extended for a long period of time, sustained and progressive changes may accumulate (a phenomenon called "allostatic load"), resulting in a substantial pathogenic deviation from the original brain functioning (*Fig. 1.3*) (*McEwen and Stellar, 1993; McEwen, 1998*).
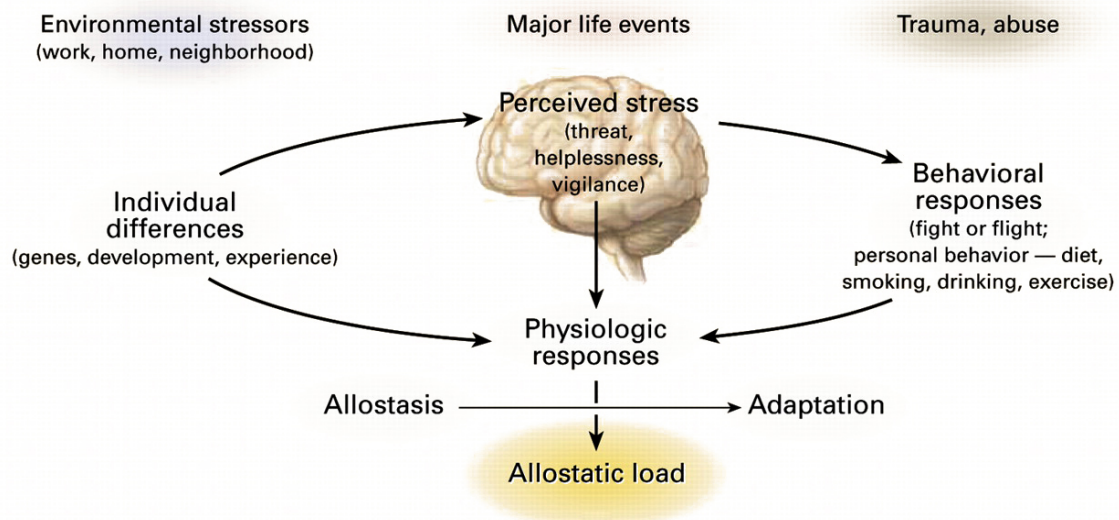
Figure 1.3: Allostasis and allostatic load in stress response
Harmful stimuli induce physiologic and behavioral responses that lead to allostasis and adaptation. Over time, however, allostatic load can accumulate, increasing the risk of disease. From *McEwen, 1998.*

The brain reacts to stress mainly by activating two mechanisms. The first is represented by the rapid activation of the autonomic nervous system, resulting in the secretion of adrenaline and noradrenaline (*McEwen et al., 1986; Joëls et al., 2012*). The two catecholamines in turn regulate body functions involved in the fight-or-flight response, including increased heart and respiratory rates, pupillary response and liberation of metabolic energy sources (*McEwen et al., 1986*).

The second mechanism by which the brain reacts to stress is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, a neuroendocrine circuit that integrates emotional, cognitive, and autonomic inputs to coordinate behavioral and hormonal responses to stress (*Fig. 1.4*) (*de Kloet et al., 2005; Lucassen et al., 2014*). The activation of the HPA axis, mainly controlled by hippocampus (HPC) and amygdala, results in increased synthesis and release of glucocorticoids from the adrenal glands (*Fig. 1.4*) (*Nestler et al., 2002; Lupien et al., 2009*). Once released, glucocorticoids (particularly

cortisol) bind two steroid receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (*de Kloet et al., 2005; Joëls and de Kloet, 2017*). Both receptors are highly expressed in brain regions involved in emotion and cognition, like HPC, amygdala and prefrontal/frontal cortex (PFC/FC), where they modulate different molecular mechanisms involved in the behavioral response to stress (*Joëls and Baram, 2009*). In particular, brain MR is constitutively activated at basal cortisol concentrations (having high affinity for glucocorticoids), is involved in learning and memory processes, in the regulation of normal neuronal functioning and acts as a quick sensor for shifts in glucocorticoid levels (*Joëls et al., 2012*). GR shows instead less affinity for cortisol than for other glucocorticoids, thus being activated by cortisol only at high stress concentrations (*Gomez-Sanchez, 2014*). Once activated, brain GR assist the brain's reaction to stressful conditions, by suppressing HPA axis and modulating MR activity (*Joëls et al., 2012*).
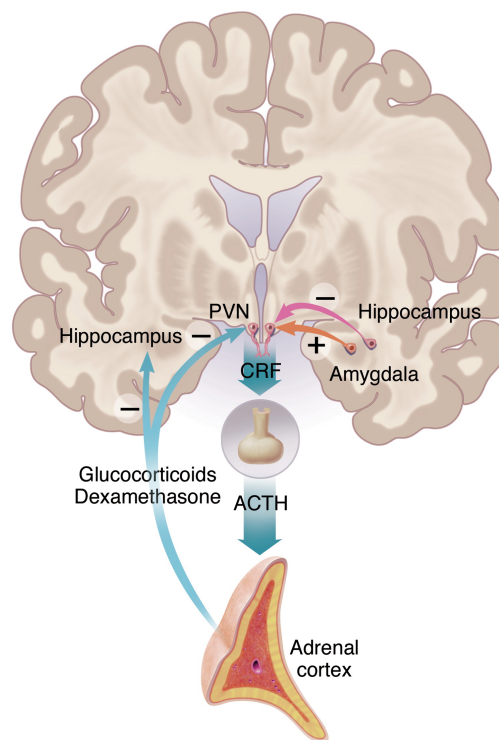
Figure 1.4: The hypothalamic–pituitary–adrenal axis
Following activation of the HPA axis, the paraventricular nucleus of the hypothalamus produces the corticotrophin-releasing hormone (CRH); CRH reaches the anterior pituitary gland, where it leads to the secretion of the adrenocorticotropin hormone (ACTH). ACTH in turn stimulates the synthesis and release of cortisol from the adrenal cortex of adrenal glands. From *Nestler et al. 2002*.

Notably, dysregulation and abnormalities in HPA axis, such as hypertrophy of the adrenals and pituitary glands, or increased plasma levels of cortisol, are estimated to be present in up to 65% of depressed patients (*Swaab et al., 2005; Lucassen et al., 2014*), confirming the central role of the HPA axis in stress response.

1.3.4 Vulnerability versus resilience

Individuals with overused or dysregulated stress response have a higher risk of developing stress-related disorders, including MDD (*Gold et al., 2015; McEwen, 2017*).

Indeed, depression may be the pathophysiological outcome of a repeated or continuous adaption to stress over the lifespan, as shown by the association between allostatic load scores and somatic/depressive symptoms in old population (*Kobrosly et al., 2014*).

Luckily, despite the strong impact of stress on health and mood, only less then 20% of the individuals exposed to prolonged stress develops a stress-related disorder, whereas most of the people maintain normal psychological and physical functioning (*Han and Nestler, 2017*). This natural ability to cope and adapt well to significant stressors is a phenomenon called resilience (*Russo et al., 2012; American Psychiatric Association, 2013; McEwen, 2016*).

Resilience has not to be considered as a simple lack of stress sensitiveness, but rather an active coping mechanism that protects the organism from the detrimental outcomes of stress (*Feder et al., 2009; McEwen, 2016*). Different psychological coping skills increasing personal resilience have been identified, including optimism, cognitive flexibility, self-esteem, or parental relationships (*Southwick et al., 2005*). Moreover, genetic findings and preclinical research on animal models are now providing consistent evidence on the biological processes underlying resilience (*Southwick et al., 2005; Russo et al., 2012; Han and Nestler, 2017*).

Depending on genetic predisposition, environmental factors, and allostatic load of stressors throughout the lifespan, the individual response to stress may take different trajectories, ranging from the complete successful recovery of homeostasis in more resilient individuals, to the onset of pathological changes in more vulnerable ones (*McEwen, 2017*). Unravelling the molecular underpinnings of stress resilience and vulnerability will be of great help in promoting the prevention and treatment of MDD and stress-related disorders (*Uher, 2008; Hughes, 2012; Musazzi et al., 2017a*).

1.4 Etiopathogenesis of major depressive disorder

The research field on the pathophysiology of MDD, as well as the development of therapeutic drugs, has been dominated for over half a century by the monoamine hypothesis of depression (*Sanacora et al., 2012*). In the last decades however, alternative theories have tried to overcome the main weaknesses of the monoamine hypothesis and to provide novel insights in the etiopathogenesis of MDD (*Hasler, 2010*).

1.4.1 The monoamine hypothesis

The monoamine hypothesis of depression postulates that deficits in monoaminergic neurotransmitters (particularly serotonin, noradrenaline and dopamine) underlie the pathophysiology of depression. The hypothesis was first formulated from the serendipitous observation that drugs able to increase the synaptic availability of monoamines produce an antidepressant effect (*Bunney et al., 1965; Frazer, 1997*). More recently, neuroimaging studies, together with analyses of monoamine metabolites levels in plasma, urine and cerebrospinal fluid of depressed patients, confirmed the dysregulation of the monoaminergic system in MDD (*Syvälahti, 1987; Meyer et al., 2006*). Additionally, experimentally induced serotonin depletion and abnormalities of serotonin receptors (*Drevets et al., 1999; Neumeister et al., 2004*), decreased noradrenaline transporter density (*Hasler, 2010*), and decreased dopamine turnover and neurotransmission (*Nutt, 2006*) have been consistently associated with altered mood and reward-related behavior.

Despite the undoubted involvement of monoamines in MDD, the monoamine-deficiency theory is characterized by relevant inconsistencies, the major being the temporal discrepancy between the immediate enhancing effect of drugs on monoamine system and their slow therapeutic benefit, which typically occurs after several weeks of chronic treatment (*Penn and Tracy, 2012; Sanacora et al., 2012*). Moreover, the side effects of monoaminergic drugs, the high percentage of non-responding patients, and the discovery of antidepressant agents that do not increase monoamine levels, have suggested that monoamine deficiency may likely be a secondary downstream effect of other and previous abnormalities (*Hasler, 2010; Penn and Tracy, 2012*).

1.4.2 The glutamate hypothesis

In the last decade, it has become increasingly acknowledged that dysfunctions of the glutamate system have a primary role in the etiology of MDD (*Sanacora et al., 2011; McCarthy et al., 2012; Musazzi et al., 2013*).

Glutamate is the major excitatory neurotransmitter in the brain, with glutamatergic neurons representing about 80% of total neurons and forming 85% of all synapses in the neocortex (*Douglas and Martin, 2007*). Under physiological conditions, glutamate neurotransmission is precisely regulated, in order to maintain optimal efficiency and limited excitotoxicity (*Niciu et al., 2012*) (*Fig. 1.5*). However, disruption of this regulation (such as changes in glutamate release, glutamate receptor expression and function, or glial cells-mediated glutamate clearance and metabolism) has been linked to the pathophysiology of stress-related disorders, including MDD (*McCarthy et al., 2012; Popoli et al., 2012*).
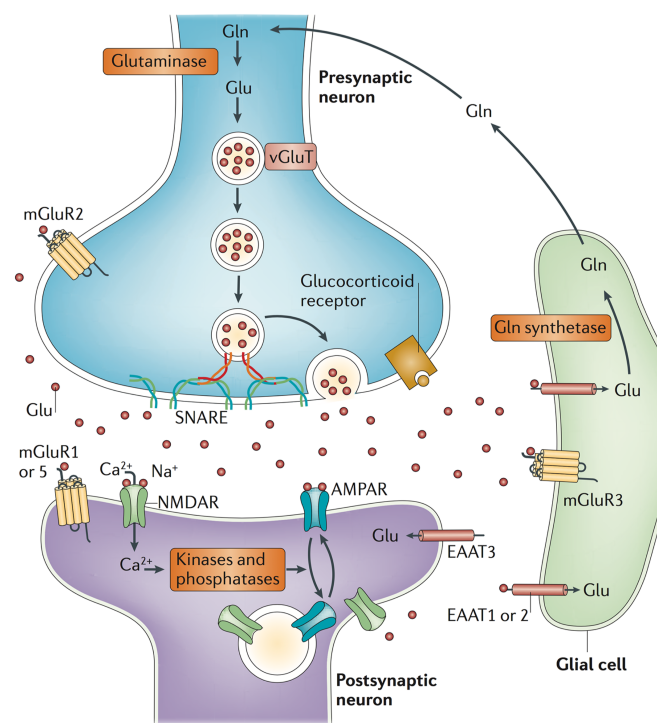
Figure 1.5: The tripartite glutamate synapse

Neuronal glutamate (Glu) is synthesized from glutamine (Gln) supplied by glial cells, and then packaged into synaptic vesicles. Fusion of vesicles with the presynaptic membrane allows glutamate release into the extracellular space. Here, glutamate binds its ionotropic and metabotropic receptors (both at pre- or post-synaptic level). Glutamate is then cleared from the synapse through excitatory amino acid transporters (EAATs) of glial cells, and converted to glutamine by glutamine synthetase. Glutamine is subsequently released by glial cells and taken up by neurons, completing the glutamate–glutamine cycle. From *Popoli et al., 2012*.

The glutamate hypothesis of depression was first suggested by the preclinical finding that antagonism of the N-methyl-D-aspartate (NMDA) receptor had a potential antidepressant effect (*Trullas and Skolnick, 1990*). Starting from this observation, a large number of studies have provided clinical evidence for the involvement of the glutamate system in MDD. In line with this hypothesis, alterations in the levels of glutamate and its metabolites have been reported in plasma (*Küçükibrahimoğlu et al., 2009*), cerebrospinal fluid (*Frye et al., 2007*) and limbic/cortical brain areas (*Hashimoto et al., 2007; Yüksel and Öngür, 2010*) of depressed patients. Moreover, changes in the expression of glutamate receptors (*Yüksel and Öngür, 2010; Niciu et al., 2014*), and of glial glutamine synthetase and glutamate transporters (EAAT1 and EAAT2) (*Rajkowska et al., 1999; Choudary et al., 2005*) were also measured in the brain regions of MDD patients, in line with a dysregulation of glutamate neurotransmission.

Importantly, neuroimaging studies on depressed patients found volume and connectivity alterations in the same cortical and limbic brain regions where abnormalities of the glutamate system have been reported (*Drevets, 2001; Koolschijn et al., 2009; Lorenzetti et al., 2009; Price and Drevets, 2010*). In particular, the regions mostly affected by structural changes were shown to be areas where glutamate neurons/synapses predominate, like HPC, PFC/FC and amygdala, with volumetric reduction being reported for HPC (*Fig. 1.6*) and PFC/FC, and volumetric enlargement being reported for amygdala (*Stockmeier et al., 2004; Price and Drevets, 2010*). Intriguingly, the involvement of these regions in the regulation of fundamental brain functions, such as cognition, emotional behavior, stress response, and memory (*Drevets, 2001*), suggests a possible link between structural changes and impaired mood and behavior.
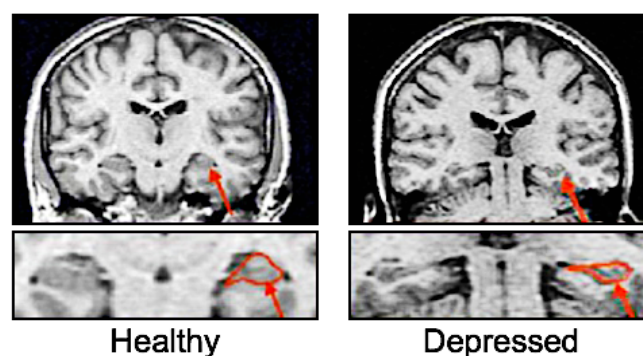
Figure 1.6: Hippocampal volume reduction in depression

Comparison of the volume of the hippocampus between a healthy control and a depressed patient. Modified from *Bremner et al., 2000.*

1.4.3 The neurotrophic hypothesis

The volumetric changes observed in the brain of depressed patients, gave support to the neurotrophic hypothesis of depression, which asserts that decrements in neurotrophic factors (particularly BDNF) have a primary role in the pathophysiology of MDD (*Krishnan and Nestler, 2008; Castrén and Rantamäki, 2010; Autry and Monteggia, 2012*). The main support for this hypothesis has come from a compelling preclinical literature, showing that BDNF-mediated signaling and structural complexity is reduced in cortical and limbic brain regions of stress-based animal models of depression (*Duman and Monteggia, 2006; McEwen et al., 2016*).

BDNF is the main neurotrophin in the adult brain, where is involved in the regulation of neuronal development and survival, synapse formation, synaptic plasticity and synaptic function (*Huang and Reichardt, 2001; Chao, 2003; Waterhouse and Xu, 2009*). Recent studies reported that BDNF protein synthesis at synapses plays a critical role in the regulation of local spines and dendritic morphology (*Verpelli et al., 2010; Baj et al., 2011; Kellner et al., 2014; Sun et al., 2014*). The distribution of BDNF mRNA at synapses have been proposed to be particularly regulated by a "spatial code" mechanism, that ensures BDNF mRNA availability, local translation and synaptic function at distinct dendritic locations (*Tongiorgi, 2008; Baj et al., 2011*). Indeed, transcription of BDNF is regulated by at least nine different promoters, each giving rise to a different splice variant transcript (*Fig. 1.7-a*) (*Sakata et al., 2009*). Among these transcripts, those containing exons 1 (BDNF-1) and 4 (BDNF-4) were shown to be more restricted to the soma and proximal dendrites, whereas those containing exon 2 (BDNF-2) and 6 (BDNF-6) were shown to extend into distal dendrites (*Fig. 1.7-b*) and regulate the local dendritic morphology (*Pattabiraman et al, 2005; Chiaruttini et al, 2008; Baj et al, 2011*).

The sorting of BDNF transcripts can be further regulated through their 3' untranslated regions (3' UTRs) (*Vicario et al., 2015*). BDNF transcript can have either short or long 3' UTRs: transcripts containing the short 3' UTR are restricted to soma, whereas the long 3' UTR mRNAs are also localized in dendrites (*An et al., 2008*).

Intriguingly, chronic treatment with conventional antidepressants is able to increase the translocation of BDNF transcripts at dendrites (*Baj et al., 2012*).
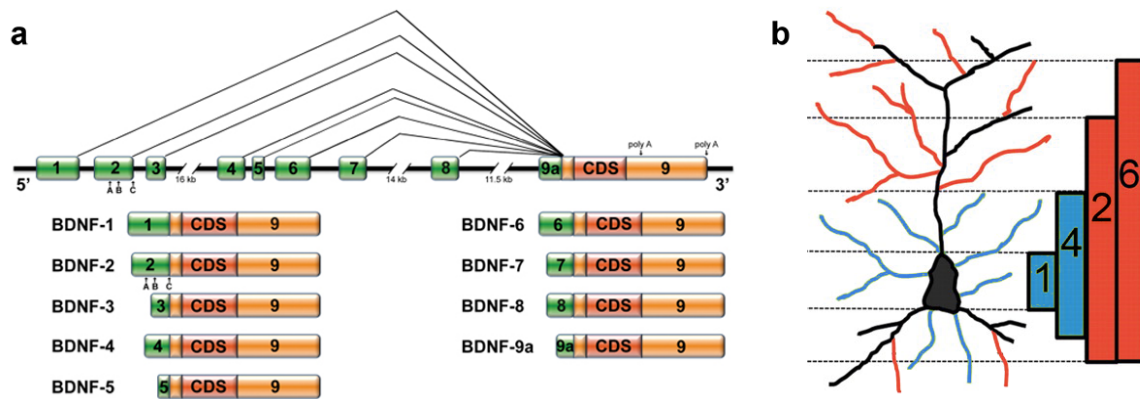
Figure 1.7: BDNF mRNA transcription and dendritic localization

(a) The BDNF gene is composed of at least nine 5' non-coding exons and one 3' coding exon; different transcript isoforms are expressed by splicing each of the non-coding exons to the coding exon. From *Mallei et al., 2015*. **(b)** Schematic representation of dendritic localization of BDNF-1, BDNF-2, BDNF-4 and BDNF-6 transcripts in a pyramidal neuron. Modified from *Baj et al., 2011*.

BDNF is also strictly interconnected with glutamatergic neurotransmission: while glutamate stimulates the production of BDNF (*Mattson, 2008*), BDNF in turn promotes both spontaneous and stimulated glutamate release (*Tyler et al., 2006; Autry and Monteggia, 2012*), and regulates the function of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptors (*Rose et al., 2004*).

Perturbations of normal BDNF regulation and levels have been associated with many pathological states, including Alzheimer's disease, Parkinson's disease, Huntington's disease, schizophrenia and MDD (*Autry and Monteggia, 2012; Vanevski and Xu, 2013*). Accordingly, brain and plasma levels of BDNF were found reduced in depressed patients (*Karege et al., 2005; Sen et al., 2008*), whereas a concomitant rescue of BDNF levels and structural changes was reported in HPC and PFC/FC, following antidepressant treatments (*Chen et al., 2001; Castrén and Rantamäki, 2010*).

1.4.4 The neuroplasticity hypothesis

More recently, the neurotrophic and glutamate hypotheses of depression have been integrated into a more comprehensive etiopathological theory, referred to as the neuroplasticity hypothesis of depression (*Sanacora et al., 2011*). According to this hypothesis, the functional and morphological changes observed in the brain of depressed patients are related to the disruption of one of the most fundamental properties of the brain: synaptic plasticity (*Pittenger and Duman, 2008; Duman et al., 2016*).

The term "synaptic plasticity" refers to the ability of the brain to sense, assess and store complex information, and to make appropriate, adaptive responses to different stimuli (*Fuchs and Flügge, 2014; Duman et al., 2016*).

Indeed, the changes in neurotrophic support, synaptic function, and remodeling of neuronal circuitry associated with MDD, suggest that neuroplasticity may be involved both in the pathophysiology of depression and in antidepressant therapy (*Racagni and Popoli, 2008; Pittenger and Duman, 2008; Sanacora et al., 2011*). As a consequence, drugs that specifically trigger synaptic plasticity should be particularly taken into account in the development of novel antidepressant treatments (*Duman et al., 2016; Gerhard et al., 2016*).

1.5 Therapeutic approaches for major depressive disorder

The standard therapeutic management of MDD involves a combination of psychotherapy and medication with antidepressant drugs (*Gelenberg et al., 2010; American Psychiatric Association, 2013; Cuijpers et al., 2014*), a class of highly heterogeneous psychotropic agents that mainly target the central nervous system (CNS), and that differ for their chemical structure, pharmacokinetics, pharmacodynamics and biochemical outcome (*Penn and Tracy, 2012*).

The treatment of depression has been revolutionized in 1960s, when two classes of drugs were discovered: the monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants (TCAs), with the former deriving from research on antitubercular drugs, and the latter arising from research on antihistamine (*Nestler et al., 2002; Racagni and Popoli, 2008*). These therapeutic agents were shown to increase the synaptic availability of monoamines, by reducing their catabolism (as for MAOIs) or by blocking their presynaptic reuptake (as for TCAs) (*Frazer, 1997*). Starting from the identification of their mechanism of action, numerous second-generation medications, with better tolerability profile, but still relevant limitations, were developed between 1980s and 1990s (*Frazer, 1997; Racagni and Popoli, 2008*). Since then, however, despite the new convincing findings on the pathophysiology of MDD, pharmacological research on antidepressants have poorly evolved (*Nestler et al., 2002*).

At present, the vast majority of the commercially available antidepressants target the monoaminergic system (*Gelenberg et al., 2010; Penn and Tracy, 2012; Sanacora et al., 2012*). A list of the antidepressant drugs most commonly used in standard clinical practice is reported in *Tab. 1.1*.

Antidepressant class	Principal mechanism of action
Monoamine oxidase inhibitors (MAOIs) *e.g. phenelzine, tranylcypromine*	Inhibition of the catabolism of serotonin, noradrenaline and dopamine
Tricyclic antidepressants (TCAs) *e.g. amitriptyline, nortriptyline, desipramine, imipramine*	Inhibition of the reuptake of serotonin and noradrenaline
Selective serotonin reuptake inhibitors (SSRIs) *e.g. citalopram, paroxetine, fluoxetine*	Selective inhibition of the reuptake of serotonin
Noradrenaline and dopamine reuptake inhibitors (NDRIs) *e.g. methylphenidate, bupropion*	Inhibition of the reuptake of noradrenaline and dopamine
Serotonin and noradrenaline reuptake inhibitors (SNRIs) *e.g. duloxetine, venlafaxine*	Inhibition of the reuptake of serotonin and noradrenaline
Serotonin antagonist and reuptake inhibitors (SARIs) *e.g. trazodone*	Antagonism of serotonin receptors and inhibition of the reuptake of serotonin
Serotonin modulator and stimulators (SMSs) *e.g. vortioxetine*	Modulation of one or more serotonin receptors and inhibition of the reuptake of serotonin
Selective noradrenaline reuptake inhibitors (NARIs) *e.g. reboxetine*	Selective inhibition of the reuptake of noradrenaline
Noradrenergic and specific serotonergic antidepressants (NaSSAs) *e.g. mirtazapine*	Antagonism of the α2-adrenergic receptor and different serotonin receptors
Melatonin receptor agonists *e.g. agomelatine*	Modulation of the melatonin and serotonin receptors.

Table 1.1: List of the most commonly used antidepressant drugs

Adapted from *Gelenberg et al., 2010* and *Penn and Tracy, 2012*.

Clinical trials have generally reported that monoaminergic therapies are, on average, 25% more effective than placebo in decreasing depressive behavior (*Walsh et al. 2002; Arroll et al. 2009*). Although the substantial benefit of such antidepressants is incontrovertible, their use is also accompanied by important limitations.

First, these drugs typically require several weeks (or even months) of chronic treatment for the appearance of a therapeutic effect (*Racagni and Popoli, 2008*), representing a particularly serious threat in those forms of depression with high risk of harm to self or suicide (*Gelenberg et al., 2010; American Psychiatric Association, 2013*). Moreover, common antidepressants frequently show relevant adverse effects, such as tachycardia, sexual dysfunction, cognitive impairment, headache, dizziness, sedation, insomnia, anxiety, fatigue, constipation, weight gain, reduced appetite, or nausea (*Gelenberg et al., 2010; Penn and Tracy, 2012*). Notably, side effects, together with the absence of noticeable therapeutic outcomes for several weeks, often dissuade patients from following appropriately (if not at all) medical prescriptions (*Weich et al., 2007*). Finally, it has been estimated that more than one-third of patients adequately treated for MDD do not respond to first-line antidepressant treatments (*Souery et al., 2007; Akil et al., 2017*); among them, 15–33% also fail to respond to two or more different antidepressants, and is identified as treatment resistant (*Cain, 2007; Gaynes et al., 2009*).

It has become increasingly acknowledged that there is an urgent need to develop better and faster pharmacological treatments for MDD. In this context, the exciting finding that ketamine induces a rapid antidepressant response, gave rise to new pharmacological research on antidepressants (*Machado-Vieira et al., 2009; Aan et al., 2012; Krystal et al., 2013; Sanacora and Schatzberg, 2015*).

1.6 Ketamine

Ketamine (KET), or 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one (*Fig 1.8*), is a synthetic phencyclidine derivative, belonging to the class of the arylcyclohexylamines, mostly used as chlorhydrate racemic mixture for its anesthetic, analgesic and (more recently) antidepressant effects (*Mion and Villevieille, 2013; Tyler et al., 2017*).
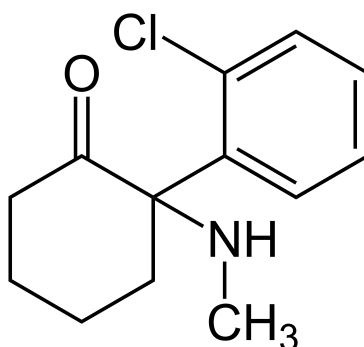
Figure 1.8: Ketamine chemical structure

KET molecule contains an asymmetric carbon atom with two enantiomers: the S(+) isomer and the R(−) isomer. Adapted from *PubChem* database of chemical molecules.

1.6.1 Pharmacology

KET acts as a non-competitive antagonist of the NMDA receptor, by binding to an allosteric intrachannel site, the so called "phencyclidine site", and resulting in decreased channel opening time and frequency (*Mion and Villevieille, 2013*). Although the antagonism of the NMDA receptor is thought to represent KET main mechanism of action, KET has been shown to also interact with AMPA, opioid, cholinergic, nicotinic, muscarinic and monoaminergic receptors (*Kohrs and Durieux, 1998; Mion and Villevieille, 2013; Tyler et al., 2017*).

KET displays low binding to plasma proteins, and high liposolubility and CNS permeability, thus reaching a high bioavailability and a rapid distribution into body tissues (*Tyler et al., 2017*). Relatively high concentrations of KET appear in body fat, liver, lung, and brain shortly after treatment (particularly following parenteral administration), while lower concentrations can be found in heart, skeletal muscle, and plasma (*Domino et al., 1984; Mion and Villevieille, 2013*).

KET is mostly metabolized into norketamine, an active metabolite that in turn is mainly hydroxylized in 6-hydroxy-norketamine (*Morris et al., 2017; Tyler et al., 2017*). KET metabolism does not simply involve the liver, but also kidneys, intestine and lungs (*Edwards and Mather, 2001; Mion and Villevieille, 2013*). Following glucuronoconjugation, KET is rapidly excreted in bile and urine, reaching an overall half-life of 2-3 hours (*Domino et al., 1984*).

1.6.2 Anesthetic, analgesic and adverse effects

KET was first synthetized by Calvin Stevens in 1962, in the attempt to generate an anesthetic agent with fewer side effects than phencyclidine (*Mion and Villevieille, 2013*). The first human studies confirmed that intravenous infusions of 1-5 mg/kg of KET were able to induce anesthesia, without affecting cardiovascular and respiratory functions, in contrast to phencyclidine (*Domino and Chodoff, 1965*). KET thus reached clinical use in 1970 (*Kurdi et al., 2014*).

Despite being a generally safe anesthetic drug, KET exhibits only less psychodysleptic effects than phencyclidine (*Corssen and Domino, 1966*). Indeed, if compared with other anesthetic drugs, KET induces a different state of anesthesia, defined as "dissociative" (*Domino, 2010*). Dissociative anesthesia causes patients to experience a dreamlike level of detachment from their body and environment during the awakening phase, with visual and auditive disturbances, and distorted perception of reality and time, lasting for a few hours (*Corssen and Domino, 1966*; *Domino, 2010*). Furthermore, high doses or too fast administration of KET were also shown to lead to acute delirium (*Mion and Villevieille, 2013*).

Due to its psychotomimetic effects, the use of KET as anesthetic has been reduced since the 1980s (*Mion and Villevieille, 2013*). Unfortunately, for the same hallucinogenic properties, KET also became a popular recreational drug, with chronic abusers showing negative outcomes on psychophysical health (*Tyler et al., 2017*). Particularly, KET abuse has been associated with cognitive impairments (*Morgan and Curran, 2012*), cortical structural and functional deficits (*Liao et al., 2010; Liao et al., 2011; Liao et al., 2012*), cystitis and biliary dilatation (*Tyler et al., 2017*).

Thanks to its peculiar anti-hyperalgesic properties, the clinical interest for KET was partly renewed during the 1990s (*Mion and Villevieille, 2013*). KET was shown to achieve

observable analgesia already at doses of 0.1−0.5 mg/kg, at which psychotomimetic risk is highly reduced, and started being effectively implemented in the treatment of chronic and post-operative pain (*Kurdi et al., 2014*).

Nowadays, KET still represents a suitable general anesthetic and an off-label analgesic medication in humans, and remains widely used in veterinary medicine (*Mion and Villevieille, 2013; Kurdi et al., 2014; Tyler et al., 2017*).

1.6.3 Ketamine as a fast antidepressant: preclinical and clinical evidence

Key observations leading to the use of KET as antidepressant initially came from preclinical studies (*Skolnick et al., 2009*). The evidence that chronic exposure to uncontrollable and inescapable stressors disrupts long-term potentiation (LTP) (*Shors et al., 1989*), a molecular mechanism of neural plasticity that is dependent on NMDA receptor (*Harris et al., 1984*), first suggested that direct modulation of NMDA receptor function could produce an antidepressant response. Accordingly, reduced NMDA receptor functionality was identified as a common outcome of classical antidepressants (*Skolnick et al., 1996*). Moreover, different antagonists of the NMDA receptor were shown to be able to mimic the effects of clinically effective antidepressants in animal models (*Trullas and Skolnick, 1990*). By the mid-1990s, several other preclinical studies suggested that NMDA receptor antagonists had antidepressant properties (*Skolnick et al., 2009*).

Preclinical literature, together with the increasing findings on the pathophysiological involvement of the glutamate system in the onset of MDD (see section 1.4.2), led Berman and collaborators to test the antidepressant efficacy of KET in humans (*Berman et al., 2000; Krystal et al., 2013*). In a placebo-controlled, double-blinded trial, 0.5 mg/kg of KET, acutely administered to MDD patients, resulted in an impressively fast and robust antidepressant effect (*Fig. 1.9*) (*Berman et al., 2000*). Even faster (1-2 hours) and long-lasting (at least 1 week) antidepressant action was reported in a subsequent study, selectively involving treatment-resistant depressed patients (*Zarate et al., 2006*).

The vast majority of clinical studies up to date, report that the antidepressant effect of a single sub-anesthetic dose of KET emerges by 2 to 4 hours after treatment (with a substantial mood improvement at 24 hours), and lasts from a few days to more than 2 weeks (*Machado-Vieira et al., 2009; Aan et al., 2012; Krystal et al., 2013*).
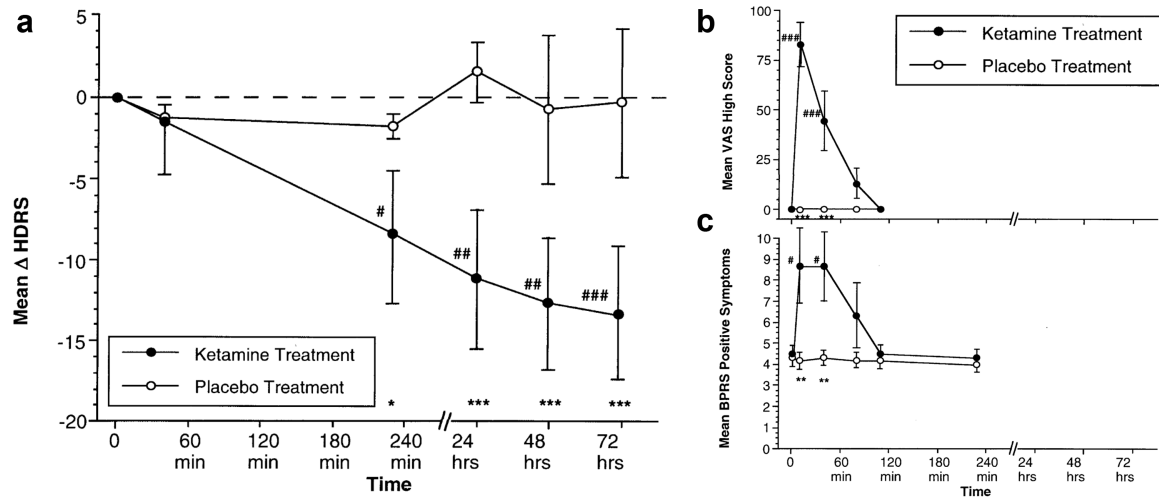
Figure 1.9: Ketamine fast antidepressant effect in depressed patients

The fast antidepressant effects of KET **(a)**, measured as variation in the Hamilton Depression Rating Scale (ΔHDRS), emerges as soon as **(b)** euphoric (VAS = visual analog scale) and **(c)** psychotigenic (BPRS = Brief Psychiatric Rating Scale) effects abate. Comparison to baseline (#) and between groups (*). Modified from *Berman et al., 2000*.

Clinical studies also suggest that antidepressant effect may be further sustained to more than 6 months by repeated KET intermittent administration (*Blier et al., 2012; Murrough et al., 2013*).

Notably, it was shown that KET directly targets the main symptoms of MDD, such as sad mood, anhedonia, helplessness and suicidality, rather than inducing a nonspecific mood-elevating effect (*Mathew et al., 2012*). Clinical benefits appear in approximately 50% to 80% of the patients (*Krystal et al., 2013*), with treatment-resistant individuals (*Zarate et al., 2006*) and patients with alcohol dependence (*Phelps et al, 2009*) or chronic pain comorbidity (*Zanicotti et al., 2012*) being particularly responsive.

At present, although a phase III trial on intranasal KET is underway (*Krystal et al., 2013*), the psychotomimetic properties of KET, together with its abuse liability, limit a wide clinical use of this drug in the therapy of MDD (*Aan et al., 2012; Sanacora and Schatzberg, 2015*).

So far, prospective follow-up data suggest that antidepressant doses of KET do not possess a long-term psychotigenic risk in healthy human subjects (*Perry et al., 2007*) and depressed patients (*Lahti et al., 2001*), including those with pre-existing psychotic features (*Ribeiro et al., 2016*). However, even at low sub-anesthetic doses, KET treatment induces transient euphoric and psychotomimetic effects in the first hours following its

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administration (*Fig. 1.9*) (*Berman et al., 2000*), thus restricting its use to clinical settings and clinical trials (*Perry et al., 2007*).

Another particular concern for KET implementation in the therapy of MDD is whether repeated administration may result in relevant adverse effects, as those observed in KET abusers (*Sanacora and Schatzberg, 2015*). Indeed, repeated daily administration in animals has been shown to produce major side effects, including deficits in glutamate and gamma-aminobutyric acid (GABA) synapses, depletion of dopamine levels, and cognitive impairment (*Braun et al., 2007; Neill et al., 2010*). However, additional studies on the safety of KET chronic treatment are still needed in humans (*Krystal et al., 2013*).

Despite KET limitations, the discovery of its fast antidepressant effect has led to a sudden increase of research on fast-acting antidepressants, raising hope for more efficient treatments (*Machado-Vieira et al., 2009; Aan et al., 2012; Krystal et al., 2013*). For this reason, KET has been repeatedly used as a powerful and promising tool to unveil the molecular basis of depression (*Fig. 1.10*) (*Wanderer and Rathmell, 2014; Sanacora and Schatzberg, 2015; Machado-Vieira et al., 2017*).
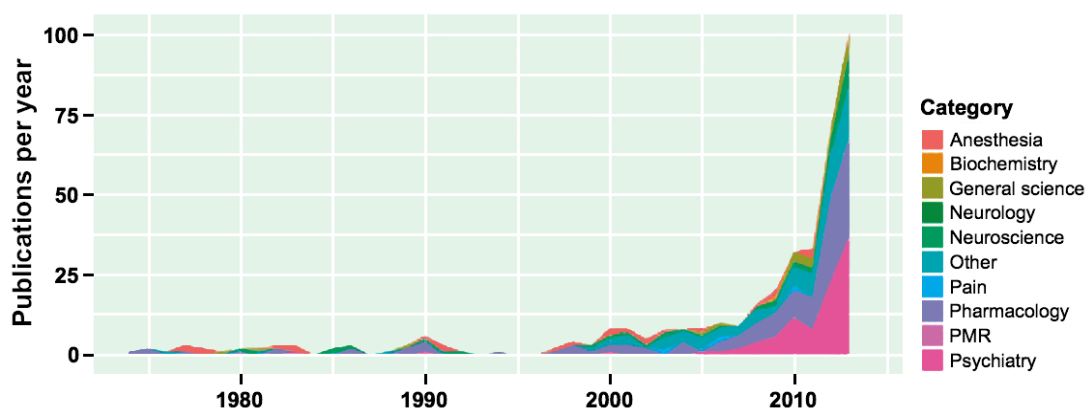
Figure 1.10: Number of publications on ketamine as antidepressant

Total number per year and category of publications on KET as antidepressant, between 1974 and 2014. From *Wanderer and Rathmell, 2014.*

1.6.4 Behavioral effects in rodents

Understanding the mechanisms underlying KET fast antidepressant action could significantly help in the development of novel, more efficient, antidepressants. To this aim, preclinical research has extensively tested the behavioral outcomes of KET in rodents, to

identify potential correlates with the antidepressant action in humans (*Browne and Lucki, 2013; Ramaker and Dulawa, 2017*). In the majority of the studies, KET have been administered intraperitoneally at sub-anesthetic doses ranging from 5 to 50 mg/kg, with 10 mg/kg being the most prevalent dose (*Browne and Lucki, 2013*).

Forced swim and tail suspension tests have repeatedly reported that acute KET administration reduces behavioral despair (*Koike et al., 2011; Wang et al., 2011*), in a similar way to chronic treatment with most of classical antidepressants (*Browne and Lucki, 2013*). Acute KET also produces fast anxiolytic effects, both in the novelty suppressed feeding test (*Carrier and Kabbai, 2013*) and in the elevated plus maze (*Engin et al., 2009*), whereas anxiolytic effects are less commonly reported with chronic conventional antidepressants (*Browne and Lucki, 2013*).

To better investigate the putative therapeutic effect of KET, many studies have also employed the use of rodent models of depression, based on the repeated exposure to behavioral stressors (*Ramaker and Dulawa, 2017*) (for a description of the most common animal models of depression, see section 1.7). Indeed, stress paradigms in rodents induce anhedonia, a core symptom of depression, that has been shown to be responsive to chronic, but not acute, treatment with classical antidepressants (*Willner, 2005*). Reports of these studies indicate that acute administration of KET restores rodent hedonic behavior within 24 hours after treatment (*Li et al., 2011*); in the same studies, antidepressant-like effects persisted from several days to more than one week after administration, thus confirming in rodents the rapid and sustained antidepressant action reported in humans (*Ramaker and Dulawa, 2017*).

1.6.5 Effect on glutamate neurotransmission

Since KET, a NMDA receptor antagonist, directly targets the glutamate system, many studies investigated the effect of KET on glutamate neurotransmission (*Abdallah et al., 2015; Monteggia and Zarate, 2015; Duman et al., 2016; Hare et al., 2017*).

Microdialysis analyses, examining KET-induced changes in glutamate release, reported that sub-anesthetic doses of KET induce a rapid and transient burst of extracellular glutamate in PFC/FC (*Moghaddam et al., 1997*). To explain this rather unexpected effect of KET on glutamate release, it has been hypothesized that KET, being an open-channel blocker, may preferentially antagonize NMDA receptors located on tonically active

inhibitory interneurons (*Farber, 1998*). Blockade of these receptors was proposed to lead to a decrease in the GABAergic inhibitory feedback, ultimately resulting in a disinhibition of excitatory networks (*Abdallah et al., 2015; Duman et al., 2016*). Accordingly, antagonism of the NMDA receptors was shown to decrease the activity of GABA interneurons and increase the firing rate of pyramidal glutamatergic neurons in layer V of the PFC/FC (*Homayoun and Moghaddam, 2007*).

Disinhibition of glutamate signaling and increase in glutamate release was shown to activate ionotropic AMPA receptors; subsequently, this results in release of BDNF and induction of signaling cascades involved in protein synthesis and neuroplasticity (*Fig. 1.11-a*) (*Abdallah et al., 2015; Duman et al., 2016*). Activation of AMPA receptors and increased ratio of AMPA- over NMDA receptor-mediated neurotransmission seem to be particularly necessary for KET antidepressant action, since antagonism of AMPA receptors blocks the behavioral effects promoted by KET (*Koike et al., 2011*).

A second hypothesis on the mechanisms underlying KET antidepressant action suggests that its fast-therapeutic onset may be the result of a direct antagonism on specific NMDA receptors regulating spontaneous glutamate neurotransmission (*Kavalali and Monteggia, 2012; Kavalali and Monteggia, 2015; Monteggia and Zarate, 2015*). While evoked neurotransmission is usually coupled to neuronal activity, spontaneous neurotransmission occurs as a result of the spontaneous fusion of a synaptic vesicle with the presynaptic membrane (*Kavalali and Monteggia, 2012*). Several studies have demonstrated that, when glutamate is released spontaneously, in the absence of presynaptic action potentials, it specifically binds to dedicated NMDA receptors, that in turn activate intracellular signaling cascades different from those involved by glutamate evoked transmission (*Atasoy et al., 2008; Espinosa and Kavalali, 2008*). Although this phenomenon is less commonly studied than evoked neurotransmission, it has been demonstrated that spontaneous neurotransmission may have critical roles in CNS, including modulation of action potential firing, maturation and stability of synaptic networks, local dendritic protein synthesis, and homeostatic synaptic plasticity (*Kavalali et al., 2011; Nosyreva et al., 2013*). Intriguingly, *in vitro* electrophysiological experiments performed in hippocampal neuron, have shown that low doses of KET inhibit NMDA receptor-mediated spontaneous miniature excitatory post-synaptic currents, suggesting that KET blocks NMDA receptors at rest (*Autry et al., 2011*). This results in specific downstream intracellular signaling, that

include the fast protein translation of BDNF and other traditional synaptic plasticity-related processes (*Fig. 1.11-b*) (*Autry et al., 2011; Kavalali and Monteggia, 2015*).
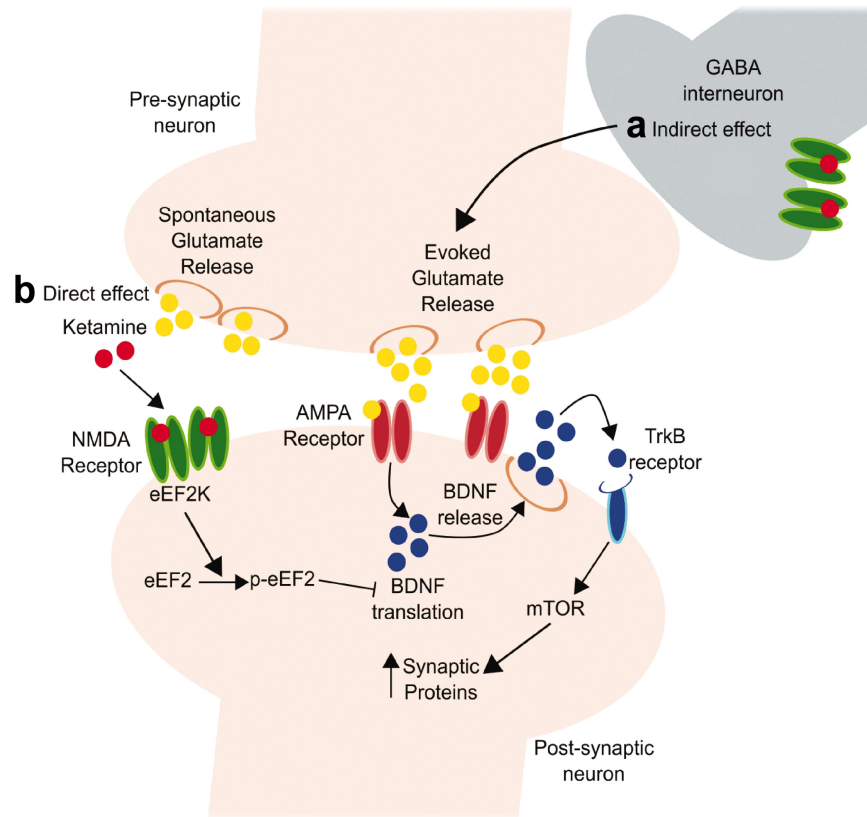
Figure 1.11: Proposed mechanisms underlying ketamine antidepressant effect
(a) The indirect hypothesis postulates that KET produces a glutamate burst via blockade of NMDA receptors located on GABAergic interneurons; glutamate burst causes activity dependent release of BDNF and activation of mTOR signaling pathway, leading to increased levels of synaptic proteins and AMPA receptor insertion. **(b)** The direct hypothesis postulates that the effects of KET occur via blockade of NMDA receptors, that are activated by spontaneous glutamate release; this results in inhibition of eEF2K and increased translation of BDNF. Modified from *Hare et al., 2017*.

1.6.6 Effect on synaptic plasticity

KET has been shown to activate several downstream signaling cascades implicated in plasticity mechanisms as well as in the pathophysiology of neuropsychiatric disorders (*Duman et al., 2016*).

One of these mechanisms is the mammalian target of rapamycin (mTOR) (*Fig. 1.11*) (*Li et al., 2010; Duman and Aghajanian, 2012*). mTOR is a serine/threonine protein kinase, which modulates fundamental cellular and neuronal functions, including cell survival, energy balance, protein synthesis, circuit formation and synaptic plasticity (*Lipton and Sahin, 2014*). Evidence of the involvement of mTOR in depression comes from

post mortem studies, which reported consistent disruption of mTOR signaling in the PFC/FC of subjects diagnosed with MDD (*Abelaira et al., 2014*). Activation of mTOR-mediated protein translation have also been demonstrated to be a critical step in the rapid antidepressant response to KET. Li and collaborators showed that KET rapidly and transiently activates mTOR, leading to a long-lasting increase in synaptic proteins and number of spines in PFC/FC (*Fig. 1.11-a*) (*Li et al., 2010*). Conversely, blockade of mTOR signaling completely prevented KET induction of synaptogenesis (*Li et al., 2010*). KET administration also results in similar rapid and transient increase in the phosphorylation of the translation initiation factor 4E-binding protein (4E-BP) and of the p70 ribosomal protein S6 kinases (p70S6K), respectively resulting in de-repression and activation of protein translation (*Li et al., 2010*). Furthermore, studies in rodents exposed to chronic stressors showed that a single dose of KET rapidly reverses the deficits in mTOR signaling pathway (*Chandran et al., 2013*), and mTOR-mediated expression of synaptic proteins (*Li et al., 2011*) induced by stress.

Given the contribution of BNDF signaling in the pathophysiology and treatment of MDD, many studies also investigated the involvement of BDNF on the rapid action of KET (*Duman and Aghajanian, 2012; Kavalali and Monteggia, 2012*). Studies in primary neuronal cultures showed that glutamate-induced AMPA receptor activation stimulates the translation and release of BDNF (*Jourdi et al., 2009*); once released, BDNF binds its receptor TrkB, which in turn activates the mTOR pathway (*Fig. 1.11*) (*Lepack et al., 2014; Lepack et al., 2016*). It was also reported that KET can rapidly stimulate the translation of BDNF, independently on mRNA transcription, via inhibition of spontaneous NMDA receptor activity (*Kavalali and Monteggia, 2012; Kavalali and Monteggia, 2015*). Indeed, blockade of NMDA receptor at rest with low doses of KET inactivates the eukaryotic elongation factor 2 kinase (eEF2K) (*Sutton et al., 2007; Monteggia et al., 2013*). This leads to activation of the eukaryotic elongation factor 2 (eEF2) and to fast de-suppression of BDNF translation at synapses (*Fig. 1.11-b*) (*Autry et al., 2011*). In line with the central role of BDNF in KET antidepressant action, BDNF deletion, TrkB or eEF2K knock-down, and BDNF neutralizing antibody were all shown to block the antidepressant effect of KET (*Autry et al., 2011; Nosyreva et al., 2013; Lepack et al., 2016*). In addition, mice carrying the human polymorphism Val66Met of the BDNF gene, do not show synaptogenic or antidepressant behavioral responses to KET (*Liu et al., 2012*).

Overall, eEF2 and mTOR signaling may cooperate in mediating the long-lasting effects on dendritic growth, spine enlargement, and synaptic protein levels promoted by KET treatment (*Li et al., 2010; Machado-Vieira et al., 2017*). In particular, it is thought that BDNF fast translation via eEF2K inhibition may initially support the BDNF release promoted by the KET-induced rapid burst of glutamate; in turn, increased BDNF release may enhance mTOR signaling, thus stimulating synapse formation and reversing the synaptic deficits caused by chronic stress exposure (*Fig. 1.11*) (*Hare et al., 2017*).

1.7 Animal models of depression

Animal models of disease represent a great tool to investigate both illness etiopathogenesis and potential therapeutic approaches. Generally, a valid animal model of a disease should accurately replicate the symptomatology as well as the molecular underpinnings of the pathology (*Nestler et al., 2010*). However, a complex pathology like human depression cannot be easily translated into animal models (*Nestler et al., 2010*).

One of the main reasons, is that there is still no clear evidence on the pathophysiological mechanisms leading to the development of depression (*Krishnan and Nestler, 2011*). Moreover, MDD is a heterogeneous pathology, associated with a wide range of phenotypes and symptoms, which cannot be entirely reproduced in animals (*Nestler et al., 2010*). MDD involves higher human brain functions, such as self-consciousness, self-reflection, motivation and cognition, leading to the impossibility to produce symptoms as for example low self-esteem, guilt or suicidality (*Krishnan and Nestler, 2011*).

Despite these limitations, several animal models have been generated in the last years, in the attempt to provide novel insights in the etiology and treatment of depression (*Willner, 2002; Hasler et al., 2004; Nestler et al., 2010; Krishnan and Nestler, 2011; Abelaira et al., 2013*). To improve their validity, selected symptoms (defined endophenotypes), such as anhedonia, behavioral despair, changes in body weight, disturbances of the HPA axis, or anxiety, have been modeled in animals (*Hasler et al., 2004*). This strategy has allowed to determine with better accuracy whether a single phenotype and its response to antidepressants might be functionally relevant to the etiology and treatment of depressive-like phenotype (*Krishnan and Nestler, 2011*).

Three criteria have been enstablished to evaluate the validity of animal models (*Willner, 2002; Abelaira et al., 2013*):

- face validity, according to which behavioral manifestations should be similar to the symptoms observed in MDD patients;

- construct validity, according to which major pathophysiological changes that occur in MDD patients, such as changes in the HPA axis, hippocampal atrophy, and levels of neurotransmitters, must also occur in animals.

- predictive validity, according to which behavioral changes should be reversed by antidepressant treatments effective in humans.

In the last years, two types of animal models have mostly been employed in the study of depression: genetic models of depression and stress-based models of depression (*Krishnan and Nestler, 2011*).

1.7.1 Genetic models

The first genetic models of depression have been developed by selecting animals, whose genetic background produced the appearance of a depressive-like phenotype (*Willner, 2002; El Yacoubi and Vaugeois, 2007; Krishnan and Nestler, 2011*).

One of the most common genetic model of depression is the Flinders Sensitive Line (FSL) rat, which is the result of a selective breeding for sensitivity to the hypothermic effect of cholinergic agonists, since increased cholinergic sensitivity has been reported in depressed patients (*Willner, 2002; Overstreet et al., 2005*). In comparison to their control Flinders Resistant Line, FSL have a number of characteristics reminiscent of depression, including anhedonia, increased cholinergic sensitivity, serotonin deficiency and increased REM sleep (*Willner, 2002; El Yacoubi and Vaugeois, 2007*). FSL animals also show greater immobility in the forced swim test and a greater vulnerability to behavioral stress (*Overstreet et al., 2005*).

Another frequently employed parameter to select genetic models of depression is the learned helplessness (LH), in which animals are selected on the basis of their impairment of escape learning, following the exposure to inescapable and uncontrollable electric shocks (*Willner, 2002; Krishnan and Nestler, 2011*). Together with a state of helplessness, LH animals also develop many symptomatic parallels to MDD, including weight loss,

alterations in sleep patterns, HPA axis hyperactivity and loss of spine synapses in hippocampal regions (*Cryan and Mombereau, 2004; El Yacoubi and Vaugeois, 2007*).

In the last years, genetic manipulation of mice genome, including gene deletion or insertion, sequence mutation or transgenic technologies have provided novel tools to generate animal models of depression (*Cryan and Mombereau, 2004*; *Krishnan and Nestler, 2011; Barkus, 2013*). Among the genetically engineered models of depression, the serotonin trasporter knock-out mice, the α2A adrenoceptor knock-out mice and the noradrenaline transporter knock-out mice have been used for their face, construct and predictive validity (*Barkus, 2013*).

1.7.2 Stress-based models

Behavioral stress is acknowledged as the main risk factor for MDD (*Kessler, 1997; Hammen, 2005; McEwen, 2013; Popoli et al., 2014; Uher, 2014b*). Accordingly, exposure to acute or chronic stressors produces a sequence of hormonal, biochemical and neuronal outcomes, that may precipitate in the onset of pathological changes typical of MDD (*Willner, 2002*). Due to the detrimental role of behavioral stress, the vast majority of currently employed animal models of depression is based on the repeated exposure to stress paradigms (*Willner, 2002; Krishnan and Nestler, 2011; Abelaira et al., 2013*).

One of the most commonly used stress-based model of depression is represented by the social defeat stress (*Krishnan and Nestler, 2011*). This paradigm is based on the emotional and psychological stress generated in a rodent by the social conflict with a dominant and aggressive rodent of the same species (*Hollis and Kabbaj, 2014*). Socially defeated rodents show a number of profound physiologic and behavioral changes, including social avoidance and alterations in the HPA axis (*Krishnan and Nestler, 2011*).

Maternal deprivation stress is instead the animal model most commonly employed to assess the predisposing effects of early-life stress in the development of MDD (*Krishnan and Nestler, 2011*). In this stress paradigm, rodent pups, in the early postnatal developmental periods, are repeatedly separated from their mothers, leading to a sustained activation of the HPA axis (*Huang et al., 2002*). This paradigm results in enduring behavioral, endocrinological, cognitive and brain-structural abnormalities, that persist through adulthood (*Krishnan and Nestler, 2011*)

In the last years, the chronic mild stress model of depression, have been one of the most widely used animal model in the study of the pathological and therapeutic mechanisms underlying MDD (*Krishnan and Nestler, 2011; Hill et al., 2012; Willner, 2017a; Willner, 2017b*).

1.8 Chronic mild stress

The chronic mild stress (CMS) model of depression is a validated model that consists in subjecting rodents to a variable sequence of mild and unpredictable stressors, thus leading to a chronic depressive-like phenotype (*Krishnan and Nestler, 2011; Hill et al., 2012; Willner, 2017a*).

1.8.1 Chronic mild stress paradigm and anhedonic behavior

The CMS paradigm originated from a series of studies by Katz and colleagues, published in the early 1980s, in which rats, sequentially exposed to a variety of severe stressors (including intense footshock, cold water immersion, or 48 hours of food and water deprivation), showed behavioral changes that were specifically reversed by chronic treatment with antidepressants (*Katz et al., 1981; Katz and Baldrighi, 1982*).

Starting from these observations, at the end of 1980s Willner and colleagues developed the CMS model of depression, by introducing two main modifications to Katz protocol (*Willner et al., 1987*). First the severity of the stressors employed was markedly reduced, in part for ethical reasons, and in part because high intensity stressors are generally more linked to the development of anxiety-like phenotypes, rather than depressive-like behavior (*Willner, 1997; Willner, 2017a*). Moreover, the assessment of the hedonic response of rodents to rewarding stimuli, measured as preference for a palatable sucrose solution, or its consumption, became the primary behavioral outcome of the model (*Willner, 2017a*).

Accordingly, starting from the first experiments on rats (*Willner et al. 1987*) or mice (*Monleon et al. 1994*), CMS have been performed by repeatedly exposing rodents to a variety of mild stressors of different nature, including physical stressors (such as food or water deprivation and forced swim), environmental stressors (such as stroboscopic light, overnight illumination, cage tilt and soiled cage) or social stressors (such as social

isolation, crowding and change of cage mate). Stressors are presented to rodents in a random, unpredictable order, thus reducing the risk of adaptation (*Nestler et al., 2010; Willner, 2017a*), for a period ranging from a few weeks to more than one month (*Hill et al., 2012; Willner, 2017a*). The effectiveness of the procedure is usually monitored by repeatedly assessing hedonic behavior (*Hill et al., 2012; Willner, 2017a*).

Two reward-based tests may generally be performed to assess hedonic behavior: the sucrose preference test (SPT) or the sucrose consumption test, both based on the natural interest of rodents for sweet taste (*Willner, 2017a*). While SPT measures the preference for a palatable sucrose solution (0.5-2%) over water (*Strekalova et al., 2011; Willner, 2017a*), sucrose consumption measures the total intake of sweet solution (*Forbes et al., 1996; Wiborg, 2013*).

Starting from CMS, several other chronic stress paradigms, employing different type of stressors, have been developed, including the chronic unpredictable stress (CUS or UCS), the chronic unpredictable mild stress (CUMS or UCMS) and chronic varied or variate stress (CVS). However, a recent meta-analysis reported no major differences in either the intensity and unpredictability of the stressors employed, or in their outcomes (*Willner, 2017b*).

Despite considerable controversy have been raised on the poor reproducibility across laboratories (*Nestler et al., 2010; Krishnan and Nestler, 2011; Willner, 2017b)*, the validity and translational potential of CMS have led to an increase of its implementation in preclinical research on MDD and antidepressant therapies (*Fig. 1.12*) (*Willner, 2017a*).
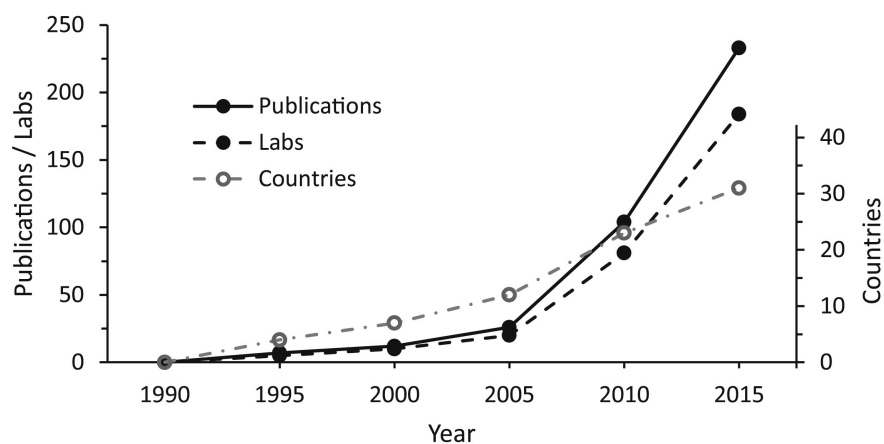
Figure 1.12: Number of publications using the chronic mild stress model of depression
Total number of publications, laboratories and countries employing the CMS model of depression each year. From *Willner, 2017a*.

1.8.2 Face, construct and predictive validity

In agreement with the face validity criteria of animal models, in the CMS paradigm rodents gradually develop behavioral disturbances comparable to those associated to human depression (*Willner, 1997*). Most of the studies reported decreased anhedonia as the main behavioral deficit induced by CMS (*Papp et al., 1996; Grippo et al., 2003; Garcia et al., 2009; Li et al., 2011; Hill et al., 2012; Willner, 2017a*). However, CMS have been shown to result in a number of other emotional changes, such as grooming deficits, changes in aggressive and sexual behavior, reduced explorative behavior and sleep disturbances (*Krishnan and Nestler, 2011; Willner, 1997*).

More recently, a further demonstration of CMS face reliability has emerged from the evidence that not all the animals exposed to CMS develop behavioral alterations (*Willner et al., 2017a*). This observation has increased the interest in examining neurochemical and individual differences in susceptibility to CMS (*Strekalova and Steinbusch, 2010; Strekalova et al., 2011; Wiborg, 2013*). Gene profiling and proteomic studies identified several hundreds of genes differently regulated in rodents vulnerable or resilient to CMS (*Bisgaard et al., 2007; Christensen et al., 2011*). Intriguingly, most of the changes regarded neurotrophins and presynaptic proteins involved in neurotransmitter release (*Bisgaard et al.,* 2007; *Henningsen et al., 2012; Han et al., 2015*).

Rodents exposed to CMS also reported many of the pathophysiological changes observed in MDD patients, confirming construct validity (*Willner, 1997; Hill et al., 2012).* Indeed, CMS exposure induces reduction in body weight and dysregulation of the HPA axis, including increased CORT and adrenocorticotropic hormone serum levels, and increased weight of adrenal glands (*Garcia et al., 2009; Hill et al., 2012*). Consistent with alterations of the HPA axis, changes in the expression of GR and MR receptors have been observed in HPC and PFC/FC of rodents following CMS, even though contrasting results were obtained between laboratories (*Hill et al., 2012*).

Exposure to CMS have been consistently associated to altered neurotransmitter levels (*Hill et al., 2012*). CMS have been shown to reduce serotonin (*Bekris et al., 2005; Vancassel et al., 2008*), noradrenaline (*Sheikh et al., 2007; Vancassel et al., 2008*), and dopamine levels (*Sheikh et al., 2007; Ahmad et al., 2010*) in HPC and PFC/FC, in line with the deficiency of monoaminergic neurotransmission reported in MDD. Reductions in GABA levels

following CMS exposure have been reported in HPC (*Grønli et al., 2007; Elizalde et al., 2010*) and PFC/FC (*Garcia-Garcia et al., 2009*).

Different studies also examined the effect of CMS on the glutamate system. Tissue analysis, performed 24 hours following the conclusion of CMS, revealed an increase of glutamate concentrations in HPC and PFC/FC (*Garcia-Garcia et al., 2009*). CMS increases the expression of the glial glutamate transporter-2 (*Raudensky and Yamamoto, 2007*), responsible for glutamate clearance from the synapse, while reduces vesicular glutamate transporter-1, responsible for the transport of glutamate into synaptic vesicles (*Elizalde et al., 2010*). CMS also impacts on the expression of ionotropic and metabotropic glutamate receptors. Indeed, reduced levels of NR2A and NR2B subunits of the NMDA receptor have been reported in HPC and PFC/FC (*Lou et al., 2010*), while reduced NR1 subunit was observed in HPC (*Fitzgerald et al., 1996*). CMS also decreases GluR1 subunit of the AMPA receptor in HPC (*Fitzgerald et al., 1996*). A decrease of the metabotropic glutamate receptors 2 (mGluR2) has been found in HPC and PFC/FC of mice subjected to CMS; however, intriguingly, the reduction of mGluR2 in HPC was found selectively in animals vulnerable to CMS, and not in resilient ones (*Nasca et al., 2015*). Furthermore, CMS increases the levels of mGluR5 in CA1 of HPC, while reduces its expression in CA3 (*Wierońska et al., 2001*).

A high number of studies also investigated the effects of CMS on BDNF mRNA and protein levels (*Hill et al., 2012*), although results do not seem to be consistent among studies. While some studies reported reduced BDNF levels in the whole HPC (*Nibuya et al., 1999; Hu et al., 2010*), no changes have also been reported (*Allaman et al, 2008*). Reduced levels of BDNF have been reported also for CA1 subregion of HPC (*Elizalde et al., 2010*), while increased levels (*Bergström et al., 2008; Larsen et al., 2010*) or no changes (*Elizalde et al. 2010*) have been found in CA3 and dentate gyrus. Concerning PFC/FC, reduced BDNF levels (*Xu et al., 2006*) or no changes (*Zhang et al., 2010*) have both been reported in rodents subjected to CMS.

More consistent effects of CMS have been reported on neuronal and brain structure (*Willner, 2017a*). In particular, atrophy of dendrites, reduced number of dendritic spines and reduced neurogenesis have been associated to CMS in the same brain areas showing volumetric reduction in MDD (*Banasr et al., 2007; Xu et al., 2007; Toth et al., 2008; Bessa et al., 2009; Li et al., 2011*).

Finally, preclinical studies have also demonstrated the predictive validity of CMS. A wide range of conventional antidepressants have been shown to reverse CMS-induced anhedonia, while several non-antidepressant agents were inactive (*Willner et al. 1987; Papp et al. 1996; Larsen et al., 2010; Zhang et al., 2010*). The reversal of anhedonia typically requires 3 to 4 weeks of treatment, closely mirroring the clinical time course of antidepressants in MDD patients (*Willner, 1997*).

Intriguingly, more recent studies using the CMS paradigm have also confirmed the fast and sustained antidepressant effect of KET (*Garcia et al., 2009; Li et al., 2011; Choi et al., 2015; Sun et al., 2016; Hare et al., 2017; Papp et al., 2017*).

2. AIMS

MDD is a highly disabling psychiatric condition which account for a large share of mental health issues worldwide and represents a great therapeutic challenge (*Fava and Kendler, 2000*). Pharmacological research on antidepressants has been dominated for over half a century by the monoamine hypothesis of depression (*Krishnan and Nestler, 2008; Hasler, 2010*). However, dysfunction of the glutamate system has been more recently indicated as one of the major players in the onset of MDD (*Sanacora et al., 2012*).

Clinical studies on depressed patients found volumetric and functional changes in glutamatergic brain areas, including HPC and PFC/FC (*Koolschijn et al., 2009*). Although the reasons for these changes have not yet been clearly identified, it has been proposed that atrophy/remodeling of dendrites and synapses has a main role in volumetric reduction (*Lorenzetti et al., 2009*). The evidence for this hypothesis comes mostly from stress paradigms in rodents, which reported atrophy, retraction, and simplification of dendritic arbor (*McEwen et al., 2016*). These changes were suggested to be linked to a maladaptive response to the abnormal glutamate transmission seen following stress exposure (*Musazzi et al., 2013*). However, while acute stress was shown to rapidly enhance glutamate neurotransmission, the impact of chronic stress on glutamate release is still largely unknown (*Popoli et al., 2012*).

Converging evidence has shown that a more direct pharmacological intervention on the glutamate system with KET induces a rapid (within hours) and sustained (up to 1 week) antidepressant effect, both in patients and in rodent models of depression (*Duman and Aghajanian, 2012*). Unfortunately, KET side effects and abuse liability limit a wide clinical use of this drug. Nevertheless, KET has been proposed as a powerful and promising tool to unveil the molecular basis of depression and of fast antidepressant treatment (*Krystal et al., 2013*). Although it has been shown that KET induces a transient

surge of glutamate release (*Moghaddam et al., 1997*), followed by an increased synthesis of BDNF (*Monteggia and Zarate, 2015*), key molecular mechanisms underlying KET therapeutic effect remain poorly understood.

Main aim of this project was to study in the CMS rat model of depression the effects of chronic stress and acute KET administration on glutamate presynaptic release, dendritic morphology and synaptic/intracellular molecular mechanisms involved in stress response.

Overall, we found major functional, molecular and morphological changes in the HPC of rats vulnerable to CMS; intriguingly, a single administration of KET was able to reverse most of these deficits.

3. MATERIALS & METHODS

3.1 Experimental plan
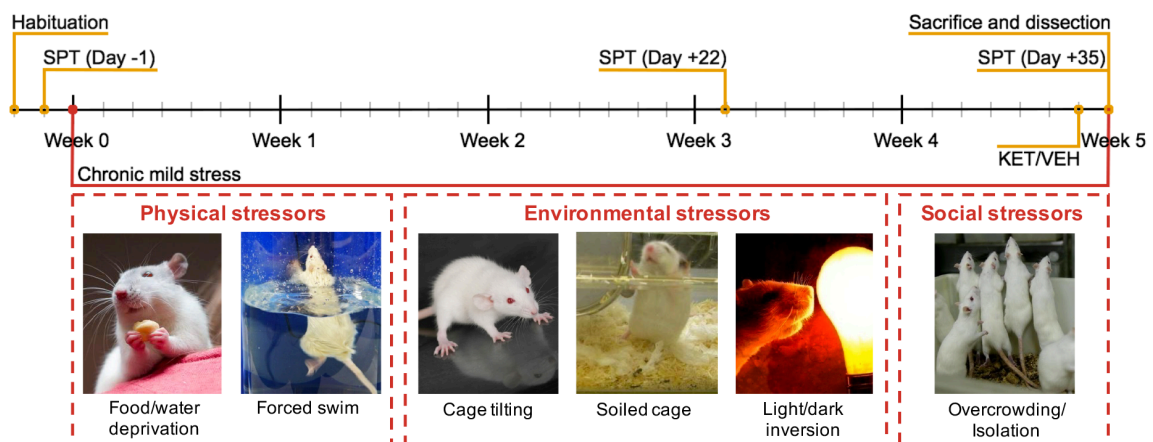
Figure 3.1: Experimental plan

Animals were subjected to a variable sequence of mild stressors for five weeks. Sucrose preference test (SPT) was performed to evaluate anhedonic behavior. KET or vehicle (VEH) were acutely administered 24 h before sacrifice.

Rats were randomly divided in two experimental groups: control (CNT; non-stressed) and CMS (stressed) groups. Two days before the beginning of CMS (Day -2), all the animals were habituated to SPT. The test was then performed the day before the beginning of CMS (Day -1), to measure basal preference for sucrose of the animals (*Fig. 3.1*).

Starting from the following day (Day 0), CMS rats were subjected to mild and unpredictable stressors, including food/water deprivation, crowding, isolation, soiled cage, cage tilting, light-on overnight, light/dark reversal and forced swim, for five weeks. Body weight gain of all animals was monitored twice a week. CNT rats were left undisturbed, except for SPT and body weighing, and kept in their home cages according to the conditions reported in section 3.2.

At the third week of CMS (Day +22), SPT was repeated; a cut-off at 55% of preference was applied to separate CMS rats in vulnerable (CMS-V) and resilient (CMS-R) animals. At the fifth week of CMS (Day +34), half of the CMS-V were injected with a single sub-anesthetic dose of KET (10 mg/kg, CMS-V+KET group), while the remaining animals received physiological solution. 23 h after the treatment (Day +35) (1 h before sacrifice) SPT was repeated. Then animals were sacrificed, HPC, PFC/FC and adrenal glands were dissected, and trunk blood was collected.

3.2 Animals

All experimental procedures involving animals were performed in accordance with the European Community Council Directive 2010/63/UE, and were approved by the Italian legislation on animal experimentation (Decreto Legislativo 26/2014, authorization N308/2015-PR).

Sprague-Dawley male rats weighing 150 to 175 g were purchased from *Charles River*. Rats were housed two per cage at 20–22° C on a 12 h light/dark cycle (light on from 7:00 a.m. to 7:00 p.m.) with water and food available ad libitum, except when required for CMS paradigm. All efforts were made to minimize animal distress and to reduce the numbers of animals used in this study.

3.3 Chronic mild stress

Rats in the CMS group were exposed once or twice daily to a random, mild and unpredictable stressor for five weeks (*Banasr et al., 2007; Li et al., 2011*) (*Fig. 3.1*). Stressors were performed at different time of the day and with a different length of time, in order to minimize prediction (*Fig. 3.2*). The following stressors were included in the CMS paradigm:

- *food deprivation*: rats were food deprived for 8-12 h;
- *water deprivation*: rats were water deprived for 8-12 h;
- *overcrowding*: rats were randomly housed five per cage for 6-12 h;

- *social isolation*: each rat was individually housed in a cage for 6-12 h;

- *soiled cage*: rats were housed in cages soiled with 500 ml of water in the sawdust, for 6-12 h;

- *cage tilting*: cages were tilted 45° left or right for 6-12 h;

- *light-on overnight*: rats were housed in a room with light turned on from 7:00 p.m. to 7:00 a.m.;

- *light/dark cycle reversal*: rats were housed in a room with inverted 12 h light/dark cycle (light off from 7:00 a.m. to 7:00 p.m.; light on from 7:00 p.m. to 7:00 a.m.)

- *forced swim*: rats were forced to swim in an upright Plexiglas cylinder (40 cm tall, 30 cm in diameter) filled with cold water (20° C), under conditions in which escape was not possible, for 5 minutes once a week.
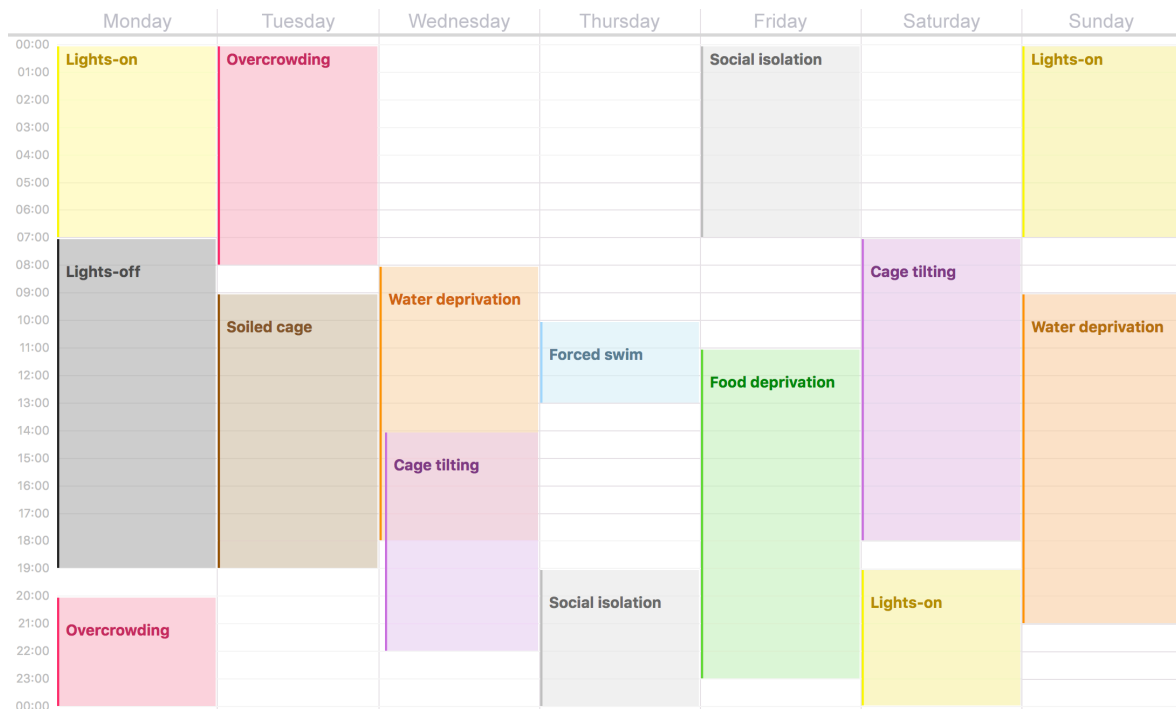
Figure 3.2: Example of a weekly schedule of CMS paradigm

The starting and duration time varied for each stressor, to avoid habituation to the paradigm.

3.4 Sucrose preference test

To validate CMS paradigm and evaluate anhedonic behavior, SPT was used (*Li et al., 2011; Strekalova et al., 2011*). Two days before the beginning of CMS, rats were first habituated to sweet taste by exposing them to two bottles, both containing 1% w/v sucrose in tap water, for 2 h. SPT was then performed at Day -1, Day +22, and Day +35 (*Fig. 3.1*). SPT consisted in presenting rats with two pre-weighed bottles, one containing 0.5% w/v sucrose solution and one containing tap water, for 1 h. The position of the two bottles was inverted halfway through the test. Bottles were weighed at the end of SPT, and the percentage of sucrose preference of each rat was calculated as:

$$\text{Sucrose preference (\%)} = \frac{\text{sucrose solution intake (ml)}}{\text{sucrose solution intake (ml) + water intake (ml)}} \times 100$$

3.5 Drug treatment

24 h before sacrifice, rats were intraperitoneally injected with a single sub-anesthetic dose (10 mg/kg) of racemic ketamine (*MSD Animal Health Srl*) or saline solution (0.9% w/v NaOH) (*Li et al., 2010; Li et al., 2011*).

3.6 Analysis of phenotypic changes

3.6.1 Body weight

Body weight gain was monitored twice a week in both CNT and CMS animals.

3.6.2 Adrenal glands relative weight

To measure adrenal glands/total body weight ratio, left and right adrenal glands were removed shortly after sacrifice, pruned from fat tissue with the help of tweezers and stereoscopic microscope, and weighed separately. The ratio was then calculated as:

$$\text{Adrenal glands relative weight (mg/g)} = \frac{\text{total weight of left + right adrenal glands (mg)}}{\text{body weight (g)}}$$

3.6.3 Corticosterone serum levels

To assess CORT levels, trunk blood was collected immediately after decapitation. Sacrifices were performed at the same time in the morning, in order to avoid differences due to CORT circadian rhythmicity. Serum was prepared from blood samples by centrifugation at 3,000 g for 20', and stored at -80° C until analysis.

CORT levels were measured using the *Corticosterone ELISA Kit* (*Enzo Life Sciences*), as previously reported (*Treccani et al., 2014; Musazzi et al., 2017b*). Briefly, the kit contains a microplate coated with donkey antibody specific to sheep IgG and a sheep anti-CORT polyclonal antibody. The test is based on the competitive binding of the anti-CORT antibody with either CORT in the samples, or synthetic CORT covalently bonded to an alkaline phosphatase molecule. The enzyme reaction was read on a *iMark Microplate Reader* (*Bio-Rad*) at 415 nm, with correction at 595 nm. CORT concentration, expressed as pg/ml, was then calculated by interpolation of the optical density of the serum sample on a CORT standard curve.

3.7 Superfusion of isolated synaptic terminals

3.7.1 Preparation of purified synaptosomes

Synaptosomes were freshly purified from homogenized HPC and PFC/FC, by centrifugation on discontinuous Percoll gradients as previously reported (*Bonanno et al., 2005; Treccani et al., 2014; Musazzi et al., 2017b*). Approximately 200 mg of HPC or PFC/FC, quickly dissected on ice immediately after sacrifice, were homogenized in 2 ml of ice-cold Homogenization Buffer (HB: 10mM Tris, 0.32 M sucrose, pH 7.4) in a glass Teflon loose-fitting potter (clearence 0.25 mm).

To remove nuclei and debris, total homogenate was centrifuged at 1,000 g for 5' at 4° C in an *Avanti$^{®}$ J-25 High Speed Centrifuge* (*Beckman*) with a *JA-20* fixed angle rotor; the surnatant (S1 fraction) was then centrifuged at 12,000 g for 5' at 4° C.

The resulting pellet (P2, a crude synaptosomal fraction) was resuspended in 600 μl of HB, gently loaded on a discontinuous Percoll (*GE Healthcare*) gradient (6%, 10%, and 20% v/v in Tris-buffered sucrose; 3 ml/step), and centrifuged at 33,500 g for 5' at 4° C. The layer between 10% and 20% steps was collected and washed with 10 ml of physiological buffer

(PB: 140 mM NaCl, 3 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM CaCl$_2$, 1.2 mM NaH$_2$PO$_4$, 5 mM NaHCO$_3$, 10 mM glucose, 10 mM HEPES, pH 7.4) by centrifugation at 13,000 g for 5' at 4° C. The resulting pellet (P2p, a purified synaptosomal fraction), was resuspended in 300 µl of PB. A 20 µl aliquot of synaptosomes was kept at -80° C for further protein concentration quantification, while the remaining sample was immediately used for the superfusion experiment.

3.7.2 Measurement of neurotransmitter release from purified synaptosomes

The method of superfusion of synaptosomes was used to measure presynaptic neurotransmitter release from purified synaptosomes (*Raiteri and Raiteri, 2000; Bonanno et al., 2005; Treccani et al., 2014; Musazzi et al., 2017b*). Freshly purified synaptosomes were layered on 25 mm mixed cellulose microporous filters (0.65 µm) (*Millipore*) at the bottom of superfusion chambers (*Ugo Basile Srl*), maintained at 37° C and superfused at a rate of 0.5 ml/min with PB (*Fig. 3.3-a/b*).
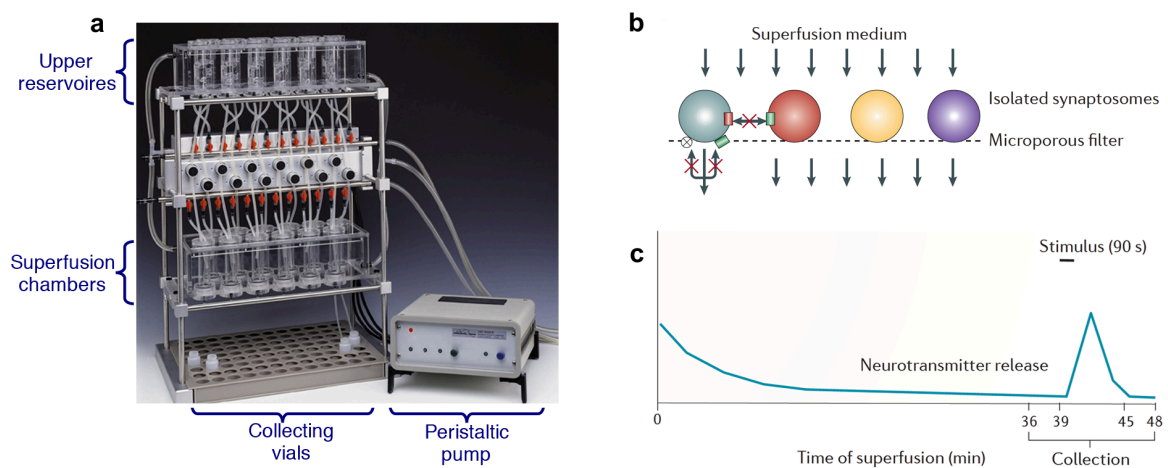
Figure 3.3: Release of endogenous neurotransmitters from purified synaptosomes

(a) Superfusion system: physiological buffer or depolarizing stimulus are loaded in the upper reservoirs; the peristaltic pump allows a constant top-down superfusion of synaptosomes contained in the superfusion chambers; released sample is collected in vials. **(b)** Synaptosomes are stratified on a monolayer in superfusion chambers; any released neurotransmitter is immediately removed by the superfusion medium, avoiding re-uptake or cross-talk between synaptosomes. **(c)** Experimental scheme: after 36 minutes of superfusion, basal neurotransmitter release is collected for 3'; following a 90'' KCl stimulus, depolarization-evoked release is collected for 6'.

After 36' of superfusion, to equilibrate the system, released sample (representing basal neurotransmitter release) was collected for 3' (t = 36–39). A 90'' synaptosomes exposure

to depolarizing concentration of KCl (15 mM), followed by 6' of collection of released sample (t = 39–45), allowed to evaluate Ca^{2+}-dependent neurotransmitter release (*Fig. 3.3-c*). Fractions collected were analyzed for endogenous glutamate, GABA and aspartate content by high-performance liquid chromatography. Amino acid release was normalized on protein concentration of the sample, and expressed as pmol/mg of protein.

3.8 Western Blot

Expression and phosphorylation levels of selected proteins were measured in different neuronal subcellular fractions of HPC and PFC/FC.

3.8.1 Preparation of homogenate, synaptosomes and synaptic membranes

- Homogenate (H): HPC or PFC/FC were homogenized as described in section 3.7.1, except for the composition of HB (10mM Tris, 0.32 M sucrose, EGTA 0.1 mM, pH 7.4). In order to protect samples from degradation, protease inhibitors (PI) (*Sigma-Aldrich*) and phosphatase inhibitors (PhI) (*Thermo Fisher Scientific*) were added to HB. 200 μl of homogenate were kept at -80° C for further assessment of protein concentration and Western Blotting, while the remaining volume was processed for synaptosomes purification.

- Synaptosomes (P2p): starting from homogenate, synaptosomes were prepared as described in section 3.7.1, with slight modifications. Percoll gradient was prepared in Tris-buffered sucrose containing 0.2 mM EDTA, 0.5 mM DTT + PI/PhI, and the P2p pellet was resuspended with 300 μl of 20 mM HEPES (pH 7.4) + PI/PhI.
  After 1' of incubation at 4° C, 150 μl of Lysis Buffer 3X (LB 3X: 360 mM NaCl, 20 mM Hepes, 0.3 mM DTT, 0.3 mM EGTA, pH 7.4 + PI/PhI) were added to lysed synaptosomes. 100 μl of synaptosomes were kept at -80° C for protein concentration quantification and Western Blotting, while the remaining volume was processed for synaptic membranes preparation.

- Synaptic membranes (LP1): synaptic membranes were prepared by adding 2 ml of Lysis Buffer 1X (LB 1X: 120 mM NaCl, 20 mM Hepes, 0.1 mM DTT, 0.1 mM EGTA, pH 7.4 + PI/PhI) to the lysed synaptosomes. Sample were then centrifuged

at 29,000 g for 30' at 4° C. The pellet (LP1 fraction, containing synaptic membranes) was resuspended in 150 µl of LB 1X, and kept at -80° C for protein concentration quantification and Western Blotting.

Protein concentration of the samples was quantified with the *Quantum Protein Assay Kit* (*EuroClone*). The kit combined the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium, with the chelation of two molecules of Bicinchoninic acid (BCA) with one Cu^{+} ion. The reaction produced was read on a *iMark Microplate Reader* (*Bio-Rad*) at 550 nm. Protein concentration of the samples was determined with reference to known concentrations of bovine serum albumin (BSA).

3.8.2 SDS-PAGE

15 µg of each sample were diluted in *2X Laemmli Sample Buffer* (65.8 mM Tris-HCl pH 6.8, 26.3% w/v glycerol, 2.1% w/v SDS, 0.01% w/v bromophenol blue, 5% v/v 2-mercaptoethanol) (*Bio-Rad*) and boiled at 100° C for 5'.

Diluted samples were loaded in handmade 10% polyacrylamide minigels (for the composition, see *Tab. 3.1*) or in *4-20% Mini-Protean TGX precast gels* (*Bio-Rad*), depending on experimental needs. Proteins were electrophoresed in a *Mini-PROTEAN II Electrophoresis Cell* (*Bio-Rad*) containing running buffer (25 mM Tris, 192 mM glycine, 0,1% w/v SDS), by applying 100 V with a *Power PAC 1000* power supply (*Bio-Rad*). Electrophoresis was controlled by using *Precision Plus Protein Dual Color* standards (*Bio-Rad*).

Running gel (10%)	Stacking gel (4%)
375 mM Tris-HCl pH 8.8	125 mM Tris-HCl pH 6.8
0.1% w/v SDS	0.1% w/v SDS
10% v/v Acrylamide-Bisacrylamide (37.5:1)	4% v/v Acrylamide-Bisacrylamide (37.5:1)
0.05% v/v Tetramethylethylenediamine	0.08% v/v Tetramethylethylenediamine
0.05% v/v Ammonium persulfate	0.1% v/v Ammonium persulfate
H_2O to final volume	H_2O to final volume

Table 3.1: Composition of running and stacking gels for Western Blotting

3.8.3 Immunoblotting

Electrophoresed proteins were transferred on polyvinylidene fluoride (PVDF) membranes (*GE Healthcare*) in a *Mini Trans-Blot Cell* (*Bio-Rad*) containing blotting buffer (25 mM Tris, 192 mM glycine, 10% v/v MeOH), by applying 200 mA for 2 h on ice, or 30 V o/n at room temperature, with a *Power PAC 1000* power supply (*Bio-Rad*). Protein transfer was controlled by reversibly staining membranes with Ponceau-S solution (*EuroClone*). Ponceau-S staining was removed by washing membranes in Tris-buffered saline containing Tween 20 (TBS-T: 20 mM Tris, 137 mM NaCl, 0.1% v/v Tween 20, pH 7.4). Prior to antibodies incubation, membranes were blocked with 5% w/v skim milk (*Serva*) in TBS-T for 1 h, to avoid non-specific binding.

For protein and phospho-protein detection, membranes were incubated o/n at 4° C or 1 h at room temperature with specific primary antibodies, diluted in TBS-T containing 5% w/v skim milk (for a list of the antibodies used and their experimental conditions, see *Tab. 3.2*); excess of primary antibody was washed 5' in TBS-T for three times. Membranes were then incubated with species-specific secondary antibodies, diluted in TBS-T containing 5% w/v skim milk, for 1 h at room temperature (for experimental conditions, see *Tab. 3.2*). Depending on experimental needs, Horseradish Peroxidase (HRP)-conjugated secondary antibodies (*Sigma-Aldrich*) or *IRDye* fluorescent-labeled secondary antibodies (*LI-COR Biosciences*) were used:

- after incubation with HRP-secondary antibodies, membranes were washed 5' in TBS-T for five times and incubated with either *ECL Star Enhanced Chemiluminescent Substrate* (*EuroClone*) for 1' or *Clarity ECL Western Blotting Substrate* (*Bio-Rad*) for 5', depending on signal intensity. The enzymatic reaction allowed to detect protein bands on a *Hyperfilm ECL* (*GE Healthcare*) chemiluminescence film in a dark room. The film was scanned on a *V700 Photo Scanner* (*Epson*) and protein bands were quantified on *Quantity One 1-D Analysis Software* (version 4.6.5) (*Bio-Rad*).
- after incubation with fluorescent-labeled secondary antibodies, membranes were washed 5' in TBS-T for five times, protecting membranes from light. Protein bands were detected on an *Odyssey Fc Imaging System* (*LI-COR Biosciences*) at 600, 700 or 800 nm, depending on the fluorophore used. Protein bands were quantified on *Image Studio* software (version 3.1.4) (*LI-COR Biosciences*).

Protein	Primary antibody	Secondary antibody
β-Actin	Mouse monoclonal (*Sigma #AB441*) 1:20000 for 1 h at RT	Goat anti-Mouse IgG (HRP or IRDye) 1:20000 for 1 h at RT
phospho-CaMKII^{Thr286}	Rabbit polyclonal (*Thermo Scientific #PA14614*) 1:1000 for 1 h at RT	Goat anti-Rabbit IgG (HRP or IRDye) 1:2000 for 1 h at RT
CaMKII	Rabbit polyclonal (*Chemicon #AB3111*) 1:1000 for 1 h at RT	Goat anti-Rabbit IgG (HRP or IRDye) 1:2000 for 1 h at RT
phospho-GR^{Ser232}	Rabbit polyclonal (*Cell Signaling #4161*) 1:1000 o/n at 4° C	Goat anti-Rabbit IgG (HRP) 1:2000 for 1 h at RT
GR	Rabbit polyclonal (*Santa Cruz #sc-1004*) 1:500 o/n at 4° C	Goat anti-Rabbit IgG (HRP) 1:1000 for 1 h at RT
mGluR2	Mouse monoclonal (*Abcam # ab15672*) 1:1000 o/n at 4° C	Goat anti-Mouse IgG (HRP or IRDye) 1:2000 for 1 h at RT
MR	Rabbit polyclonal (*Santa Cruz #sc-114112*) 1:500 o/n at 4° C	Goat anti-Rabbit IgG (HRP) 1:1000 for 1 h at RT
phospho-mTOR^{Ser2448}	Rabbit polyclonal (*Cell Signaling #2971*) 1:1000 o/n at 4° C	Goat anti-Rabbit IgG (HRP or IRDye) 1:2000 for 1 h at RT
mTOR	Rabbit polyclonal (*Cell Signaling #2972*) 1:1000 o/n at 4° C	Goat anti-Rabbit IgG (HRP or IRDye) 1:2000 for 1 h at RT
phospho-Synapsin I^{Ser9}	Rabbit polyclonal (*Cell Signaling #2311*) 1:1000 o/n at 4° C	Goat anti-Rabbit IgG (HRP or IRDye) 1:2000 for 1 h at RT
Synapsin I	Mouse monoclonal (*Synaptic Systems #106 001*) 1:4000 for 1 h at RT	Goat anti-Mouse IgG (HRP or IRDye) 1:8000 for 1 h at RT

Table 3.2: List and conditions of antibodies used for Western Blotting

To detect more than one protein with similar molecular weight on the same membrane, antibodies with different detection techniques, different fluorophores or raised from different species were used. When these strategies were not applicable, membranes were stripped by incubation in stripping buffer (63 mM Tris-HCl pH 6.8, 2% w/v SDS, 5% v/v 2-mercaptoethanol) for 30' at 55° C, and blocked with 5% w/v skim milk in TBS-T for 1 h, prior reprobing with a different antibody. All protein bands used were within a linear range and normalized for β-actin levels in the same membrane. Data were expressed as percentage of control levels.

3.9 RNA Isolation, RT-PCR and Real-Time PCR

3.9.1 RNA Isolation

Dissected HPC and PFC/FC were immediately frozen in ethanol dry ice bath and kept at -80° C until further extraction of RNA.

Total RNA was extracted from frozen HPC and PFC/FC using the *Direct-zol RNA MiniPrep* (*Zymo Research*), according to manufacturer's instructions (*Ieraci et al., 2016*). 100 mg of frozen HPC and PFC/FC were added 1 ml of *Tri-Reagent* (*Sigma-Aldrich*) and homogenized with steel beads in a *TissueLyser LT* (*Qiagen*) at 50 Hz for 3'. Homogenized tissue was centrifuged in a *5415R Micro-Centrifuge* (*Eppendorf*) at 12,000 g for 10' at 4° C. To allow protein-nucleic acid phase separation, 200 μl chloroform were added to the RNA-containing surnatant. The sample was kept 15' at room temperature and then centrifuged 12,000 g for 15'. One volume of the aqueous phase, containing RNA, was mixed with one volume of EtOH 99%; the mixture was loaded on a *Zymo-Spin IIC Column* in a collection tube and centrifuged for 1' at 12,000 g, to ensure RNA binding to the column. The column was then incubated with 80 μl of *DNase I* 15' at room temperature, to digest DNase. Following two washes with *Direct-zol RNA PreWash Buffer* (12,000 g for 1') and one wash with *Direct-zol RNA Wash Buffer* (12,000 g for 1'), the RNA bound to the column was eluted with 50 μl of DNase/RNase-free water. The concentration of the eluted RNA was measured on a *NanoVue Plus Spectrophotometer* (*GE Healthcare*).

3.9.2 Reverse Transcription

Total RNA was reverse-transcribed to cDNA using the *iScript cDNA Synthesis Kit* (*Bio-Rad*), containing oligo(dT) and random hexamer primers (*Mallei et al., 2015; Ieraci et al., 2016*). For each sample, the following reaction mix was composed on ice: 1 μg of extracted RNA; 4 μl of *5x iScript reaction mix*; 1 μl of *iScript reverse transcriptase*; nuclease-free water, to reach 20 μl of final reaction volume.

The complete reaction mix was incubated on a *Mastercycler epGradient* (*Eppendorf*) as follows: 5' at 25º C; 30' at 42º C; 10' at 50° C; 5' at 85º C; hold at 4º C.

3.9.3 Quantitative Real-Time polymerase chain reaction

Quantitative Real-Time PCR (qPCR) was performed by taking advantage of SYBR-Green (*Bio-Rad*) fluorescence (*Mallei et al., 2015; Ieraci et al., 2016*). For each well of a 96-multiwell (*Thermo Fisher*), the following reaction mix was composed on ice: 5 µl *SYBR-Green*; 1 µM of forward + reverse primers (list of primers is reported in *Tab. 3.3*); 4 µl of cDNA, diluted 1:20 in nuclease free-water.

The reaction was performed on a *7900HT Fast PCR System* (*Applied Biosystems*). PCR cycling conditions were: 10' at 95º C; 40 cycles of: 15'' at 95º C, 40'' at 55° C and 30'' at 72° C.

Relative expression of mRNA for the target genes was performed applying the comparative C_T ($\Delta\Delta C_T$) method, using P0 and SD18 as control reference genes. The relative mRNA levels were expressed as fold change.

Gene	Primer
Total BDNF	*Forward:* 5'-GGCCCAACGAAGAAAACCAT-3'
	Reverse: 5'-CAGAAAGAGCAGAGGAGGCT-3'
BDNF-1	*Forward:* 5'-AGCAAAGCCACAATGTTCCA-3'
	Reverse: 5'-CAGCCTTCATGCAACCGAAG-3'
BDNF-2	*Forward:* 5'-GGCGAGCAGAGTCCATTCAG-3'
	Reverse: 5'-GGATGAAGTACTACCACCTCGG-3'
BDNF-4	*Forward:* 5'-GAGCAGCTGCCTTGATGTTTA-3'
	Reverse: 5'-CAGCCTTCATGCAACCGAAG-3'
BDNF-6	*Forward:* 5'-GGAGCGTGACAACAATGTGA-3'
	Reverse: 5'-CAGCCTTCATGCAACCGAAG-3'
P0	*Forward:* 5'-AGTCGGAGGAATCCGATGAG-3'
	Reverse: 5'-ATTAAGCAGGCTGACTTGGTG-3'
SD18	*Forward:* 5'-CATGCAGAACCCACGACAAT-3'
	Reverse: 5'-CTTCCCATCCTTCACGTCCT-3'

Table 3.3: List of primers used for qPCR

Primers were acquired from *Eurofins Genomics*.

3.10 In situ hybridization

3.10.1 Riboprobes preparation

Digoxigenin (DIG)-labeled riboprobes detecting total BDNF, BDNF-2 and BDNF-6 transcripts were generated from PCR templates adapted with SP6 and T7 RNA polymerase sites. Riboprobes were transcribed using a *DIG RNA Labeling Kit* (*Thermo Scientific*), according to the manufacturer's instructions. Specifically, the 20 μl transcription mixture included 200 ng of template purified cDNA, 1X Transcription Buffer, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.17 mM UTP, 0.33 mM DIG-UTP (*Life Technologies*), and 40 U of either T7 or SP6 RNA polymerase. The reaction was incubated for 2 h at 37° C and stopped by adding 0.5 M EDTA. The riboprobes were then purified in *NucAway Spin Columns* (*Ambion*) and quantified with *NanoDrop 1000* (*Thermo Scientific*).

3.10.2 Brain and slices preparation

Freshly dissected left or right hemispheres, rapidly rinsed in in phosphate-buffered saline (PBS: 5.3 mM KH_2PO_4, 770 mM NaCl, 15 mM NaH_2PO_4, pH 7.4), were fixed with 4% w/v paraformaldehyde (PFA) (*Sigma-Aldrich*) in PBS, and kept at 4° C (*Baj et al, 2012; La Via et al., 2013*). After 24 h, hemispheres were moved on fresh 4% PFA in PBS and kept at 4° C. After 3 days, hemispheres were moved on PBS containing 30% sucrose, and kept at 4° C until slices preparation. Hemisphere sections of 40 μm were coronally sliced on a cryostat.

3.10.3 In situ hybridization

In situ hybridization was performed using the *Vectastain Elite ABC-Peroxidase Staining Kit* (*Vector Laboratories*) (*La Via et al., 2013*). Free-floating sections were post-fixed for 3 h in 4% PFA, washed twice in PBS containing 0.1% v/v Tween 20 (PBS-T) for 5', and treated with 0.3% v/v H_2O_2 for 20', to block endogenous peroxidase. Following three washes in PBS-T for 5', slices were permeabilized with 2.3% w/v $NaIO_4$ for 5' and quickly washed in H_2O. Sections were then incubated in 1% w/v $NaBH_4$ in 0.1 M Tris-HCl buffer (pH 7.5) for 10', and washed twice in PBS-T for 3'. The slices were digested with 8 μg/ml proteinase K (*Sigma-Aldrich*) in PBS-T for 20' and washed twice in PBS-T for 5'. After

digestion, tissue slices were fixed in 4% PFA for 5' and washed three times in PBS-T for 10'. Slices were incubated at 55° C for 90' in pre-hybridization solution, containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1X Denhardt's solution (*Invitrogen*), 300 mM NaCl, 100 mM DTT, 0.5 mg/ml salmon sperm DNA (*Gibco*), 0.5 mg/ml polyadenylic acid (*Sigma-Aldrich*), and 50% v/v formamide. Slices were incubated o/n at 55° C in hybridization solution, composed of the pre-hybridization solution supplemented with 10% dextran sulfhate and 100 ng/ml DIG-labeled riboprobes. The following day, slices were washed: twice in 2X SSC-T (saline sodium citrate containing 0.1% v/v Tween 20), supplemented with 50% v/v formamide, at 55° C for 30'; once in 2X SSC-T at 55° C for 20'; twice in 0.2X SSC-T at 60° C for 30'. *In situ* hybridization signal was obtained using biotinylated donkey anti-mouse IgG antibodies and diaminobenzidine as chromogen (*Vector Laboratories*). Sections were mounted and images of pyramidal neurons from CA1 and CA3 hippocampal regions and layer V of PFC were captured on an *Olympus BX50* microscope (*Fig. 3.4*). The maximal distance of dendritic labeling was determined on *AxioVision SE64* software (version 4.9.19, *Zeiss*) using the function "Measure Length". *In situ* hybridization analysis was performed by a blinded experimenter.
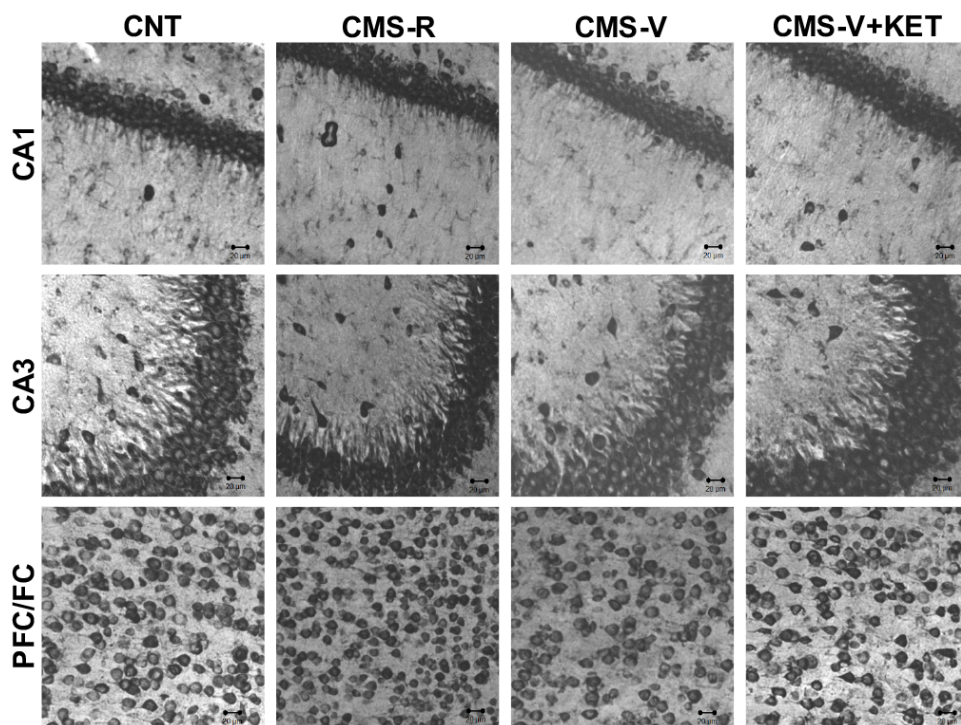
Figure 3.4: *In situ* hybridization in CA1, CA3 and PFC/FC

Representative images of *in situ* hybridization of total BDNF mRNA in CA1 and CA3 regions of HPC and in layer V of PFC/FC in CNT, CMS-R, CMS-V and CMS-V+KET rats.

3.11 Golgi staining and dendritic analysis

3.11.1 Gelatine coating of histology slides

Racks of *Menzel Glaser Superfrost* microscope slides (76x26x1 mm) (*Thermo Scientific*) were cleaned twice for 10'' in EtOH 99% and let dry o/n in a dust-free area. 1.3% w/v Gelatine (*Fluka*) and 0.13% w/v $KCr(SO_4)_2$ were completely dissolved in H_2O at 60° C. Clean slides were dipped twice in warm (40-50° C) gelatin solution for 10'', let dry o/n in a dust-free area and stored in a dust-free container at room temperature.

3.11.2 Golgi-Cox impregnation

Golgi-Cox staining was performed using the *Rapid Golgi Stain Kit* (*FD NeuroTechnologies*) (*Nava et al., 2015; Chen F et al., 2016*). Immediately after sacrifice, left or right hemispheres were rapidly rinsed in PBS and then immersed in impregnation solution (prepared by mixing equal volumes of Solution A and Solution B, at least 24 h before sacrifice) at room temperature in the dark. The impregnation solution was replaced after 24 h of immersion; hemispheres were kept in the impregnation solution for other 13 days, for a total of 14 days. Hemispheres were then kept in Solution C for at least 4 days (by replacing Solution C after the first 24 h).

3.11.3 Preparation and mounting of Golgi stained sections

Hemispheres were embedded in 6% w/v Agarose (*VWR*). Sections of 200 μm were coronally sliced on a *VT1200S* vibratome (*Leica*) in PBS and mounted on 1.3% gelatin-coated slides. Each section was covered with one drop of Solution C and kept 4' in the dark. Solution C was completely removed and sections were left drying under hood for 1 h in the dark. Racks of slides were washed twice in H_2O for 4' and then stained in Solution D+E (prepared by mixing 1 volume of Solution D, 1 volume of Solution E and 2 volumes of H_2O) for 10'. Excess of Soultion D+E was washed twice in H_2O for 4'. Sections were then dehydrated, by dipping them twice in EtOH 70%, once in EtOH 96% and thrice in EtOH 99% (4' for each step). Finally, after dipping sections in Xylene (*VWR*) three times for 4', slides were coverslipped with *Deckglaser #0* coverslips (24x50 mm) and *Eukitt Quick-hardening Mounting Medium* (*Sigma*).

3.11.4 Neuron acquisition and Imaris reconstruction

The CA3 area of HPC was identified on a *BX50* light microscope (*Olympus*) using *newCAST* software (*Visiopharm*) (*Fig. 3.5*). Pyramidal neurons were identified by dendrites extending into 2 distinct conical arbors, an apical and a basal one (*Spruston, 2008; Conrad et al, 2017*).

Figure 3.5: Localization of CA3 pyramidal neurons in Golgi-stained HPC sections
Golgi-stained HPC sections compared with Paxinos *Rat Brain Atlas*.

Z-stacks (100-130 μm; Z-step size 1 μm) of apical and basal dendrites of CA3 pyramidal neurons with untruncated branches were acquired using a ×60 oil objective. Collapsed Z-stacks were imported in *Bitplane Imaris* software (version 7.7.1) (*Andor Technology Ltd*) and dendrites were reconstructed using the "FilamentTracer" function, with "Autopath" algorithm (*Fig. 3.5-a/b*). Dendrite length and branching were calculated by choosing the soma of the reconstructed neuron as starting point, and by setting 1 μm as thinnest diameter. Sholl analysis was performed with a 20 μm radius interval, starting from the soma. For spine quantification, the thinnest diameter was set at 0.4 μm, while maximum length was set at 4 μm (*Fig. 3.5-c*). Spines were classified with the "Classify Spines" function, using *MathWorks MATLAB* software (*Fig. 3.5-d*). Morphological analysis was performed in a blinded manner.

Figure 3.6: Imaris resconstruction of a CA3 pyramidal neuron
(a) Basal and **(b)** apical dendrites of CA3 pyramidal neurons. **(c)** Dendritic spines were detected and **(d)** classified in stubby, mushroom, long thin and filopodia subtypes.

3.12 Statistical analysis

Statistical analysis was carried out using *GraphPad Prism 6* software.

For SPT (Day +22 and +35), adrenal glands relative weight, CORT serum levels, neurotransmitter release experiments, Western Blots, qPCR and morphological analysis one-way ANOVA followed by the Tukey's post-hoc test was used. For SPT (Day -1 and Day +22) unpaired two-tailed Student t-test was used. For weight gain and Sholl analysis two-way ANOVA followed by the Tukey's post-hoc test was used. For *in situ* hybridization of BDNF transcripts multilevel covariance analysis was used; two random effects were modelled, rat and slice within rat and dendritic trafficking was modelled on log-scale. *p*-values were adjusted for multiple comparison using single step procedure. Data were expressed as mean ± standard error of the mean.

4. RESULTS

4.1 Validation of the CMS protocol

4.1.1 SPT allowed to separate vulnerable from resilient rats. KET reversed anhedonic behavior

Since chronic stress paradigms in rodents are known to induce anhedonia, a core symptom of human depression (*Nestler and Hyman, 2010*), we validated CMS by performing the SPT, a standard behavioral test for anhedonia (*Li et al., 2011; Strekalova et al., 2011*). As expected, while SPT performed the day before CMS start showed no preexisting differences in preference for sucrose between CNT and CMS groups ($F_{81,104}$=1.11, p=0.8563, unpaired t-test) (*Fig. 4.1-a*), a significant reduction in sucrose preference was found in the CMS group after 3 weeks of stress ($F_{86,104}$=2.04, p<0.001, unpaired t-test) (*Fig. 4.1-b*). To separate stress-resilient from stress-vulnerable rats (*Strekalova et al., 2011*), we applied a cut-off at 55% of sucrose preference: rats showing a preference for sucrose higher than 55% were classified as resilient to CMS (CMS-R), while rats, showing preference lower than 55%, were classified as anhedonic and vulnerable (CMS-V) ($F_{2,115}$=143.9, p<0.001, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT p<0.001; CMS-V vs. CMS-R p<0.001) (*Fig. 4.1-c*).

After 5 weeks of CMS, half of the CMS-V rats were injected with a single dose of KET (CMS-V+KET), and SPT was repeated 24 h later, immediately before sacrifice. We found that, while CMS-V still showed anhedonic behavior ($F_{3,105}$=18.45, p<0.001, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT p<0.001; CMS-V vs. CMS-R p<0.001), KET treatment restored sucrose preference in CMS-V+KET (CMS-V+KET vs. CNT p<0.05; CMS-V+KET vs. CMS-V p<0.001) (*Fig. 4.1-d*).
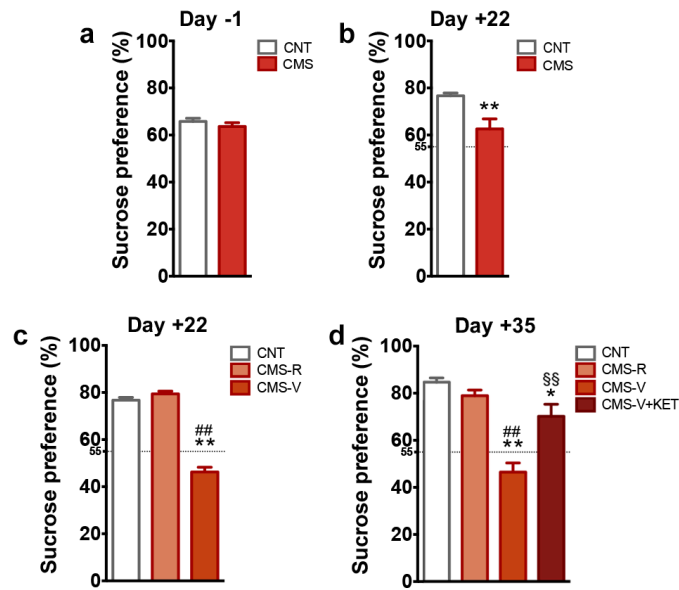
Figure 4.1: Sucrose preference test

Sucrose preference of CNT and CMS rats **(a)** one day before the beginning of CMS paradigm and **(b)** at day 22 of CMS. **(c)** Separation of resilient and vulnerable animals applying a cut-off at 55% of sucrose preference at day 22 of CMS. **(d)** SPT at day 35 of CMS, 24 hours after KET/vehicle treatment. Data are shown as means ± SEM. n = 25-30. *p<0.05 vs CNT; **p<0.001 vs CNT; ##p<0.001 vs CMS-R; §§p<0.001 vs CMS-V.

4.1.2 CMS induced stronger phenotypic changes in vulnerable rats

CMS was also validated by analyzing three different parameters known to be affected by chronic stress: body weight gain, adrenal glands relative weight and corticosterone (CORT) serum levels (*Garcia et al., 2009; Chen J. et al, 2016*).

Two-way ANOVA revealed a significant effect of CMS ($F_{2,1036}$=264.7, $p<0.001$) and time ($F_{11,1036}$=1518, $p<0.001$) on body weight gain, with a significant interaction between CMS and time ($F_{22,1036}$=8.17, $p<0.001$) (*Fig. 4.2-a*). Moreover, Tukey's *post hoc* test showed that, starting from the fifth day of stress, CMS significantly decreased body weight gain in both CMS-R and CMS-V in comparison to CNT.

Adrenal glands relative weight was found increased in CMS-V, but not in CMS-R, while KET had no significant effect ($F_{3,144} = 8.42$, $p<0.001$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT $p<0.001$; CMS-V+KET vs. CNT $p<0.001$) (*Fig. 4.2-b*).

Accordingly, the increase of serum CORT levels induced by stress was higher in CMS-V, than in CMS-R rats, and was not affected by KET (*Fig. 4.2-c*) ($F_{3,77}$=21.52, $p<0.001$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.05$; CMS-V vs. CMS-R $p<0.001$; CMS-V+KET vs. CNT $p<0.001$; CMS-V+KET vs. CMS-R $p<0.001$).
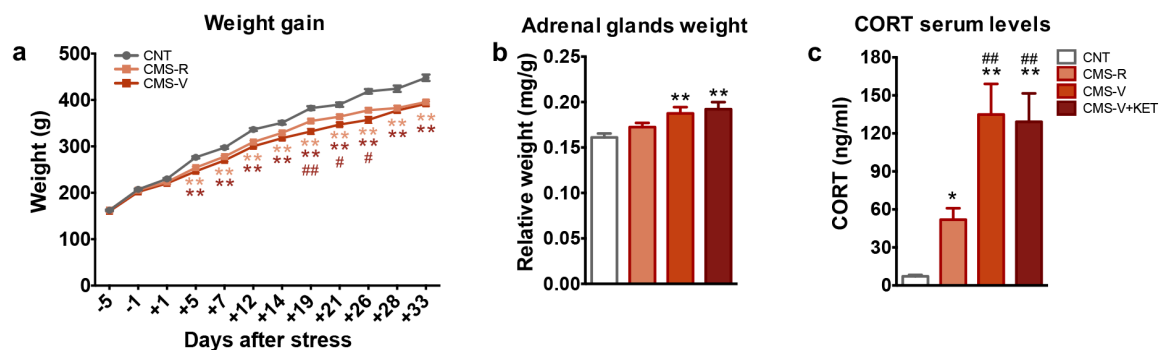
Figure 4.2: Phenotypic changes induced by CMS

(a) Body weight gain; n = 25-30. **p<0.001 CMS-R vs CNT; **p<0.001 CMS-V vs CNT; #p<0.05 CMS-V vs CMS-R ##p<0.001 CMS-V vs CMS-R. **(b)** Adrenal glands relative weight (n = 25-30) and **(c)** CORT serum levels (n = 15-20). *p<0.05 vs CNT; **p<0.001 vs CNT; ##p<0.001 vs CMS-R.

4.2 Effect of CMS and KET on endogenous presynaptic neurotransmitter release

While acute stress has been shown to induce a rapid enhancement of glutamate release and transmission (*Reznikov et al., 2007; Yuen et al., 2009; Treccani et al., 2014*), an effect blocked by chronic antidepressants (*Musazzi et al., 2010*), the impact of chronic stress and KET on glutamate neurotransmission is still largely unknown (*Popoli et al., 2012*). To better understand the effect of CMS and KET on the presynaptic release of amino acid neurotransmitters in HPC and PFC/FC, we measured the endogenous release of glutamate, GABA and aspartate using the technique of purified synaptosomes in superfusion (*Bonanno et al., 2005; Musazzi et al., 2017b*).

4.2.1 CMS altered presynaptic neurotransmitter release in HPC. KET modulated these changes

In HPC, CMS significantly reduced basal glutamate release from superfused synaptosomes of CMS-V, compared to both CNT and CMS-R ($F_{3,43} = 2.18$, $p = 0.1042$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT $p<0.05$; CMS-V vs. CMS-R $p<0.05$) (*Fig. 4.3-a*). Interestingly, Tukey's *post hoc* test revealed no significant difference between CNT and CMS-V+KET or CMS-R and CMS-V+KET, suggesting that KET partially rescues the CMS-induced reduction of basal glutamate release in CMS-V. HPC synaptosomes from CMS-V also showed reduced depolarization-evoked release of glutamate compared to CMS-R, an effect that was not restored by KET ($F_{3,37} = 4.00$, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CMS-R $p<0.05$; CMS-V+KET vs. CMS-R $p<0.001$) (*Fig. 4.3-b*).

A significant decrease in basal aspartate release was also found in both CMS-R and CMS-V compared to CNT; KET treatment was able to completely rescue this change in CMS-V ($F_{3,40} = 3.94$, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.05$; CNT vs CMS-V $p<0.001$; CMS-V vs CMS-V+KET $p<0.05$) (*Fig. 4.3-c*). Tukey's *post hoc* test showed a significant decrease of depolarization-evoked aspartate release in CMS-V+KET in comparison with CMS-R ($F_{3,36} = 2.48$, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT $p = 0.1041$; CMS-V vs. CMS-R $p = 0.0710$; CMS-V+KET vs. CMS-R $p<0.05$); a non-significant trend towards decrease was also

found in CMS-V (*Fig. 4.3-d*). Presynaptic GABA basal release was found increased in synaptosomes from CMS-R compared to CNT, while decreased in CMS-V and CMS-V+KET compared to CMS-R ($F_{3,56} = 6.10$, *p*<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT *p*<0.001; CMS-V vs. CMS-R *p*<0.05; CMS-V+KET vs. CMS-R *p*<0.05) (*Fig. 4.3-e*). Conversely, we found that CMS reduced GABA evoked release selectively in CMS-V compared to CNT, an effect that was restored to CNT levels by KET ($F_{3,30} = 2.58$, *p*<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT *p*<0.05; CMS-V+KET vs. CMS-V *p*<0.05) (*Fig. 4.3-f*).
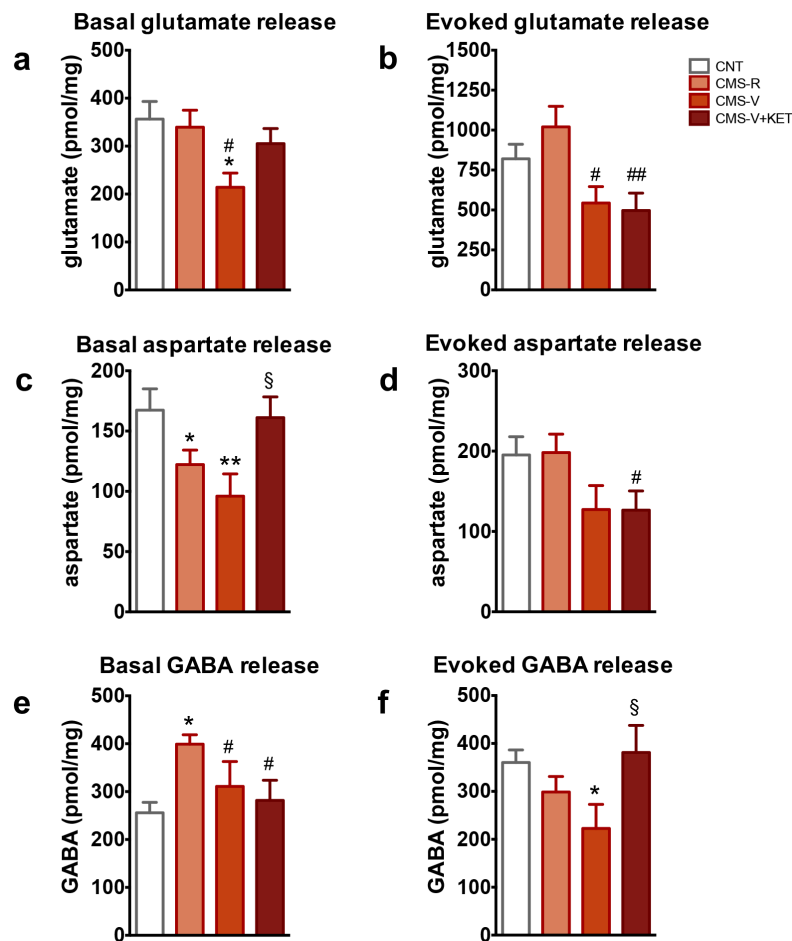
Figure 4.3: Neurotransmitter release from HPC synaptosomes in superfusion

Effect of CMS and KET on **(a)** basal and **(b)** depolarization-evoked glutamate release, **(c)** basal and **(d)** depolarization-evoked aspartate release and **(e)** basal and **(f)** depolarization-evoked GABA release. n = 8-12. *p<0.05 vs CNT; **p<0.001 vs CNT; #p<0.05 vs CMS-R; ##p<0.001 vs CMS-R; §p<0.05 vs CMS-V.

4.2.2 Presynaptic glutamate release was reduced in PFC/FC of resilient rats

In contrast with the decrease of glutamate presynaptic release observed in HPC synaptosomes from CMS-V, no changes were measured in PFC/FC of CMS-V and

CMS-V+KET. However, selectively in synaptosomes from CMS-R PFC/FC, we found a significant decrease of both basal ($F_{3,34}$ = 2.89, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.05$) (*Fig. 4.4-a*), and depolarization-evoked release of glutamate ($F_{3,40}$ = 2.90, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.05$; CMS-V vs. CMS-R $p<0.05$) (*Fig. 4.4-b*). No significant changes between experimental groups were found in the overall levels of basal aspartate release ($F_{3,38}$ = 0.26, p = 0.8546, one-way ANOVA), evoked aspartate release ($F_{3,39}$ = 1.11, p = 0.3554, one-way ANOVA), basal GABA release ($F_{3,60}$ = 1.23, p = 0.3056, one-way ANOVA) and evoked GABA release ($F_{3,35}$ = 0.63, p = 0.5997, one-way ANOVA) (*Fig. 4.4-c-f*).
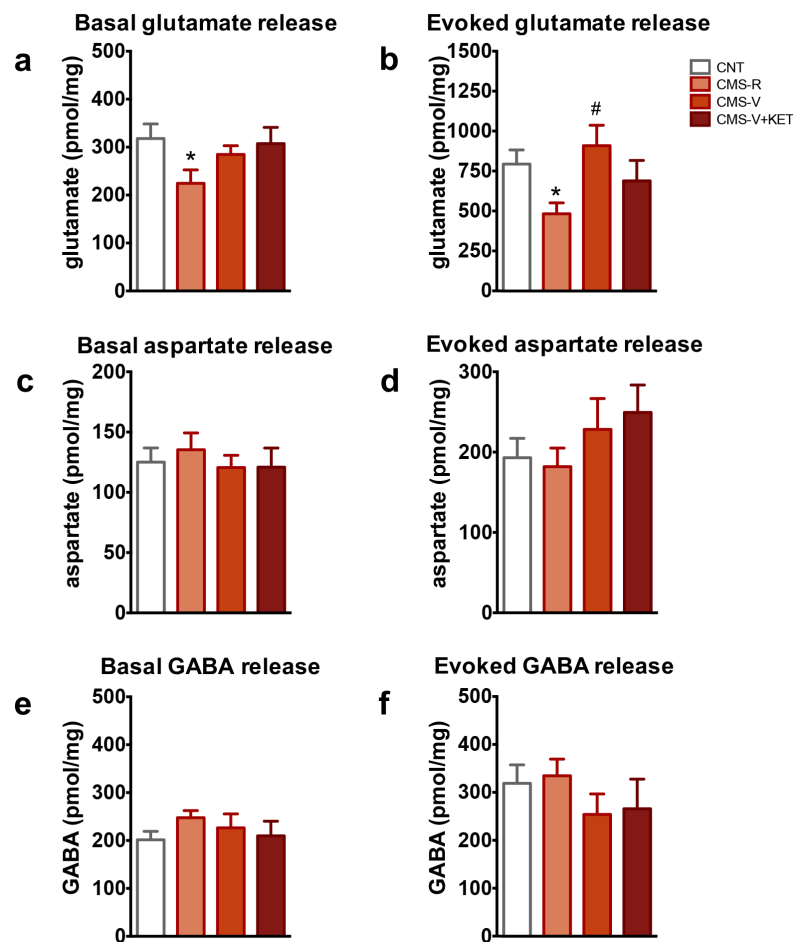
Figure 4.4: Neurotransmitter release from PFC/FC synaptosomes in superfusion

Effect of CMS and KET on **(a)** basal and **(b)** depolarization-evoked glutamate release, **(c)** basal and **(d)** depolarization-evoked aspartate release, **(e)** basal and **(f)** depolarization-evoked GABA release. n = 8-12. *$p<0.05$ vs CNT; #$p<0.05$ vs CMS-R.

4.3 Effect of CMS and KET on corticosterone receptors

We then tried to understand whether the increased circulating levels of CORT in CMS-R and CMS-V could affect the expression and function of CORT brain receptors. To do so, the expression levels of the mineralocorticoid receptor (MR) and the expression and phosphorylation levels of the glucocorticoid receptor (GR) at Ser^{232} were measured in total homogenate, purified synaptosomes and synaptic membranes from HPC and PFC/FC.

4.3.1 CMS increased synaptic localization of MR in HPC and PFC/FC

While no changes were found in the total levels of MR in HPC homogenate ($F_{3,45}$ = 1.20, p = 0.3208, one-way ANOVA) (*Fig. 4.5-a*), the receptor was found significantly increased in HPC synaptosomes from CMS-V ($F_{3,44}$ = 4.07, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT $p<0.05$; CMS-V vs. CMS-R $p<0.05$) (*Fig. 4.5-b*), indicating an enrichment of MR at synaptic level. Interestingly, Tukey's *post hoc* test revealed no significant difference between CNT and CMS-V+KET (CMS-V vs. CNT p = 0.5782), suggesting that KET partly blocked this increase. No significant changes were instead found in synaptic membranes from HPC ($F_{3,31}$ = 1.95, p = 0.1415, one-way ANOVA) (*Fig. 4.5-c*).

Similarly to HPC, CMS and KET had no effect on the total levels of MR in PFC/FC homogenate ($F_{3,41}$ = 1.70, p = 0.1815, one-way ANOVA) (*Fig. 4.5-d*), but we found increased expression of the receptor in synaptosomes from CMS-V ($F_{3,44}$ = 4.53, $p<0.01$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT $p<0.01$; CMS-V vs. CMS-R $p<0.05$; CMS-V+KET vs. CMS-V $p<0.05$), an effect which was completely rescued by KET (CMS-V+KET vs. CMS-V $p<0.05$) (*Fig. 4.5-e*). No changes were found in PFC/FC synaptic membranes ($F_{3,36}$ = 1.10, $p<0.05$, one-way ANOVA) (*Fig. 4.5-f*).
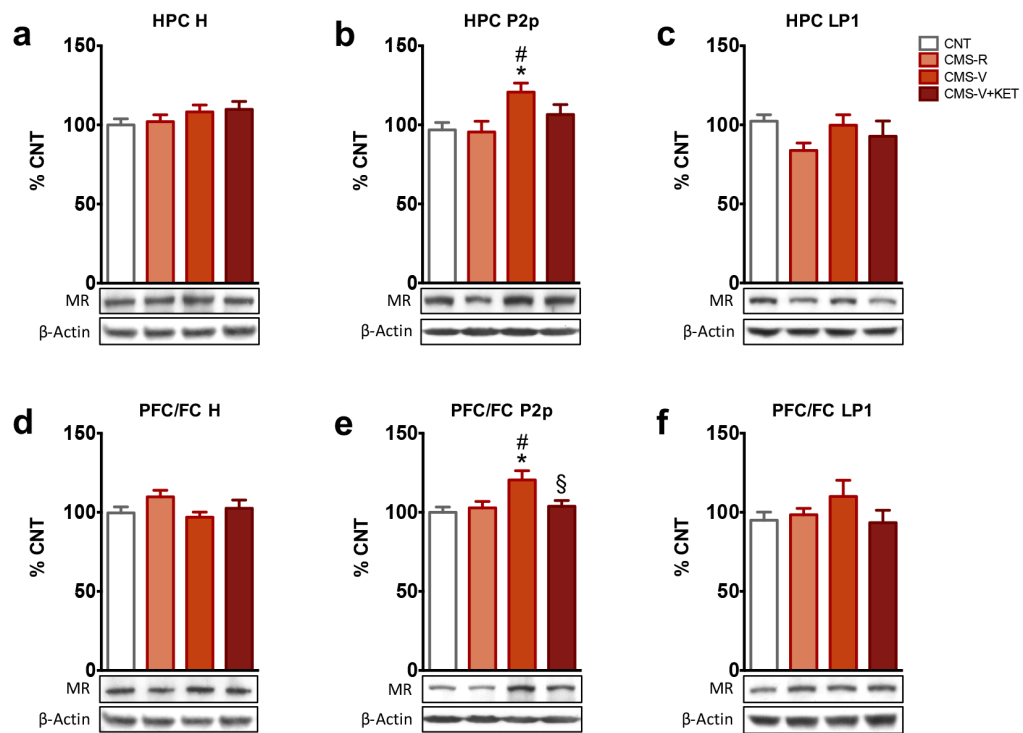
Figure 4.5: MR protein levels

Expression of MR protein on **(a)** homogenate, **(b)** synaptosomes and **(c)** synaptic membranes from HPC. Expression of MR protein on **(d)** homogenate, **(e)** synaptosomes and **(f)** synaptic membranes from PFC/FC. n = 10-12. *p<0.05 vs CNT; #p<0.05 vs CMS-R; §p<0.05 vs CMS-V.

4.3.2 KET increased the phosphorylation of GR at Ser^{232} in HPC and PFC/FC

We found no changes in GR total expression in HPC homogenate ($F_{3,45}$ = 1.41, p = 0.2517, one-way ANOVA), synaptosomes ($F_{3,38}$ = 0.25, p = 0.8608, one-way ANOVA) and synaptic membranes ($F_{3,33}$ = 0.81, p = 0.4946, one-way ANOVA), and in PFC/FC homogenate ($F_{3,45}$ = 0. 67, p = 0.5730, one-way ANOVA), synaptosomes ($F_{3,38}$ = 0.25, p = 0.8608, one-way ANOVA) and synaptic membranes ($F_{3,32}$ = 1.56, p = 0.2176, one-way ANOVA) (*Fig. 4.6*).
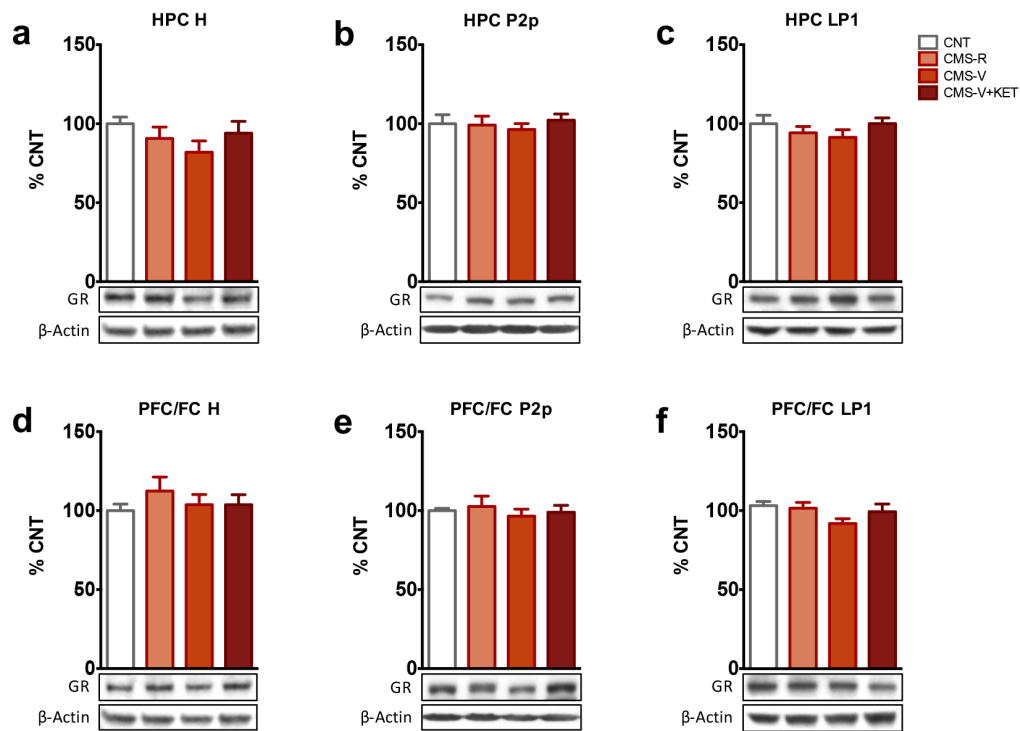
Figure 4.6: GR protein levels

Expression of GR protein on **(a)** homogenate, **(b)** synaptosomes and **(c)** synaptic membranes from HPC. Expression of GR protein on **(d)** homogenate, **(e)** synaptosomes and **(f)** synaptic membranes from PFC/FC. n = 8-12.

We also analyzed the levels of GR phosphorylated at Ser^{232} (corresponding to the human phosphorylation at Ser^{211}), a marker of enhanced GR trafficking to the nucleus and increased transcriptional activity (*Wang et al., 2002; Cattaneo and Riva, 2016*).

A significant increase of GR phosphorylation was found in HPC homogenate from KET treated rats ($F_{3,73}$ = 3.17, p<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-V+KET vs. CMS-R p<0.05; CMS-V+KET vs. CMS-V p<0.05) (*Fig. 4.7-a*). No changes were instead found in synaptosomes ($F_{3,36}$ = 0.61, p = 0.6091, one-way ANOVA) and synaptic membranes ($F_{3,35}$ = 0.36, p = 0.7818, one-way ANOVA) (*Fig. 4.7-b/c*).

Similar changes were observed in PFC/FC: CMS-V+KET rats showed increased levels of phospho-Ser[232] GR in total homogenate ($F_{3,42} = 3.89$, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-V+KET vs. CNT $p<0.05$; CMS-V+KET vs. CMS-V $p<0.05$) (*Fig.-4.7-d*), while no effects of CMS and KET were found in synaptosomes ($F_{3,38} = 0.37$, $p = 0.7750$, one-way ANOVA) and synaptic membranes ($F_{3,36} = 0.74$, $p = 0.5323$, one-way ANOVA) (*Fig. 4.7-e/f*).
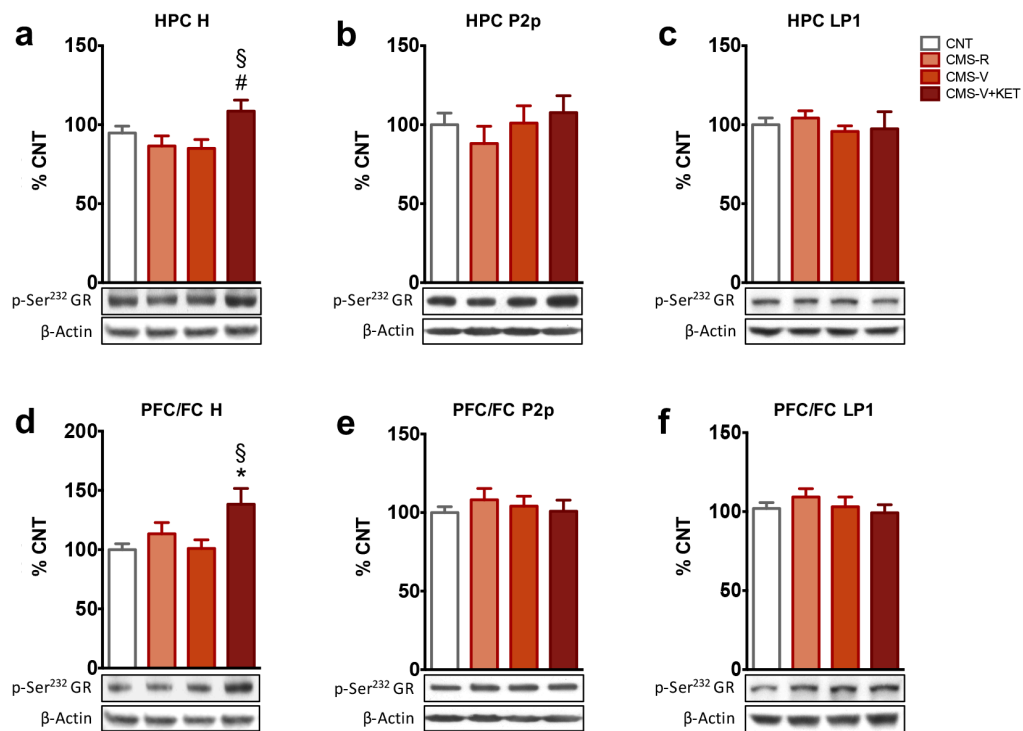
Figure 4.7: phospho-Ser[232] GR protein levels

Expression of phospho-Ser[232] GR protein on **(a)** homogenate, **(b)** synaptosomes and **(c)** synaptic membranes from HPC. Expression of phospho-Ser[232] GR protein on **(d)** homogenate, **(e)** synaptosomes and **(f)** synaptic membranes from PFC/FC. n = 8-12. *p<0.05 vs CNT; #p<0.05 vs CMS-R; §p<0.05 vs CMS-V.

4.4 Effect of CMS and KET on presynaptic proteins regulating glutamate release

In order to further characterize the impact of CMS and KET on presynaptic release of glutamate, we measured the expression levels of two presynaptic proteins involved in the regulation of glutamate release: Synapsin I and the metabotropic glutamate receptor 2 (mGluR2).

4.4.1 CMS increased the phosphorylation of Synapsin I at Ser^9 in PFC/FC of resilient rats

Synapsin I is a presynaptic protein involved in vesicle mobilization (*Cesca et al., 2010*). We previously found that acute stress increases its phosphorylation at Ser^9 in PFC/FC synaptic membranes, an event which is required for the stress-induced enhancement of the readily releasable pool size of synaptic vesicles (*Treccani et al., 2014; Musazzi et al., 2017b*).

Concerning the total levels of Synapsin I, we found no significant changes in synaptosomes ($F_{3,42}$ = 0.27, p = 0.8437, one-way ANOVA) and synaptic membranes ($F_{3,35}$ = 0.64, p = 0.5912, one-way ANOVA) of HPC, and in synaptosomes ($F_{3,39}$ = 0.60, p = 0.6138, one-way ANOVA) and synaptic membranes ($F_{3,38}$ = 0.77, p = 0.5167, one-way ANOVA) of PFC (*Fig. 4.8*).

No effect of CMS and KET was found on the phosphorylation of Synapsin I at Ser^9 in HPC synaptosomes ($F_{3,38}$ = 0.59, p = 0.6225, one-way ANOVA) and synaptic membranes ($F_{3,33}$ = 0.53, p = 0.6622, one-way ANOVA) (*Fig. 4.9-a/b*). However, CMS-R rats showed significantly increased levels of phospho-Ser^9 Synapsin I in synaptosomal fraction ($F_{3,38}$ = 3.96, p<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT p<0.05; CMS-V+KET vs. CMS-R p<0.05) (*Fig. 4.9-c*), and in synaptic membranes ($F_{3,42}$ = 4.82, p<0.01, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT p<0.05) compared to CNT; increased phospho-Ser^9 Synapsin I was also found in synaptic membranes from CMS-V+KET (CMS-V+KET vs. CNT p<0.01) (*Fig. 4.9-d*).
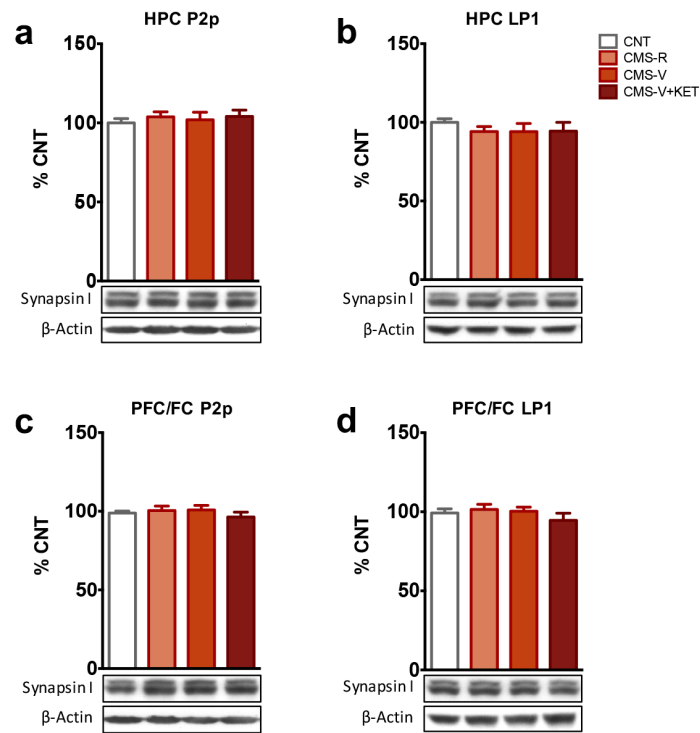
Figure 4.8: Synapsin I protein levels

Expression of Synapsin I protein on **(a)** synaptosomes and **(b)** synaptic membranes from HPC. Expression of Synapsin I protein on **(c)** synaptosomes and **(d)** synaptic membranes from PFC/FC. n = 8-12.
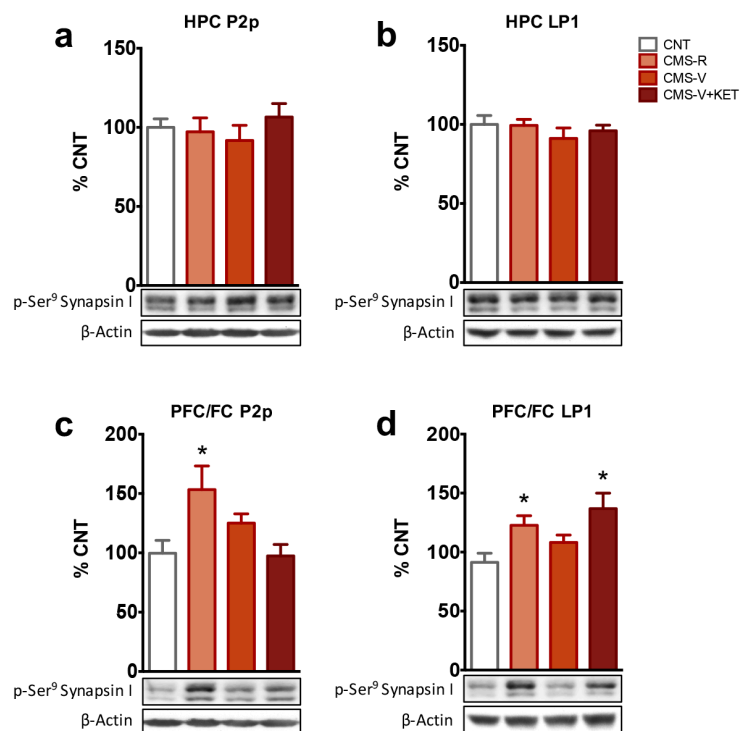
Figure 4.9: phospho-Ser^9 Synapsin I protein levels

Expression of phospho-Ser^9 Synapsin I protein on **(a)** synaptosomes and **(b)** synaptic membranes from HPC, and **(c)** synaptosomes and **(d)** synaptic membranes from PFC/FC. n = 8-12. *p<0.05 vs CNT.

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4.4.2 mGluR2 levels were reduced in vulnerable rats and restored by KET

The metabotropic glutamate receptor 2 (mGluR2) is a presynaptic receptor which negatively modulate glutamate release (*Chaki et al., 2013*). Interestingly, the receptor has recently gained attention as promising target of novel antidepressants (*Chaki et al., 2013; Bruno et al., 2017*) and for its role in stress-vulnerability (*Nasca et al., 2015*).

Our results show a significant decrease in the levels of mGluR2 in homogenate from HPC of CMS-V, while KET treatment completely rescued its expression ($F_{3,49}$ = 3.78, p<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT p<0.05; CMS-V vs. CMS-R p<0.05; CMS-V+KET vs. CMS-V p<0.05) (*Fig. 4.10-a*). mGluR2 expression was also significantly reduced in HPC synaptosomes ($F_{3,39}$ = 3.14, p<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT p<0.05), and synaptic membranes, where KET restored this change ($F_{3,29}$ = 5.55, p<0.01, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT p<0.01; CMS-V+KET vs. CMS-V p<0.05) (*Fig. 4.10-b/c*).

Similar results were found in PFC/FC, were we observed decreased mGluR2 levels in total homogenate from PFC/FC of CMS-V, an effect completely rescued by KET ($F_{3,40}$ = 10.21, p<0.001, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT p<0.01; CMS-V vs. CMS-R p<0.01; CMS-V+KET vs. CMS-V p<0.001) (*Fig. 4.10-d*). At the same time, mGluR2 was found decreased also in PFC/FC synaptosomal fraction ($F_{3,40}$ = 4.05, p<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT p<0.05; CMS-V vs. CMS-R p<0.05), and synaptic membranes ($F_{3,34}$ = 3.25, p<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CMS-R p<0.05) from CMS-V (*Fig. 4.10-e/f*).
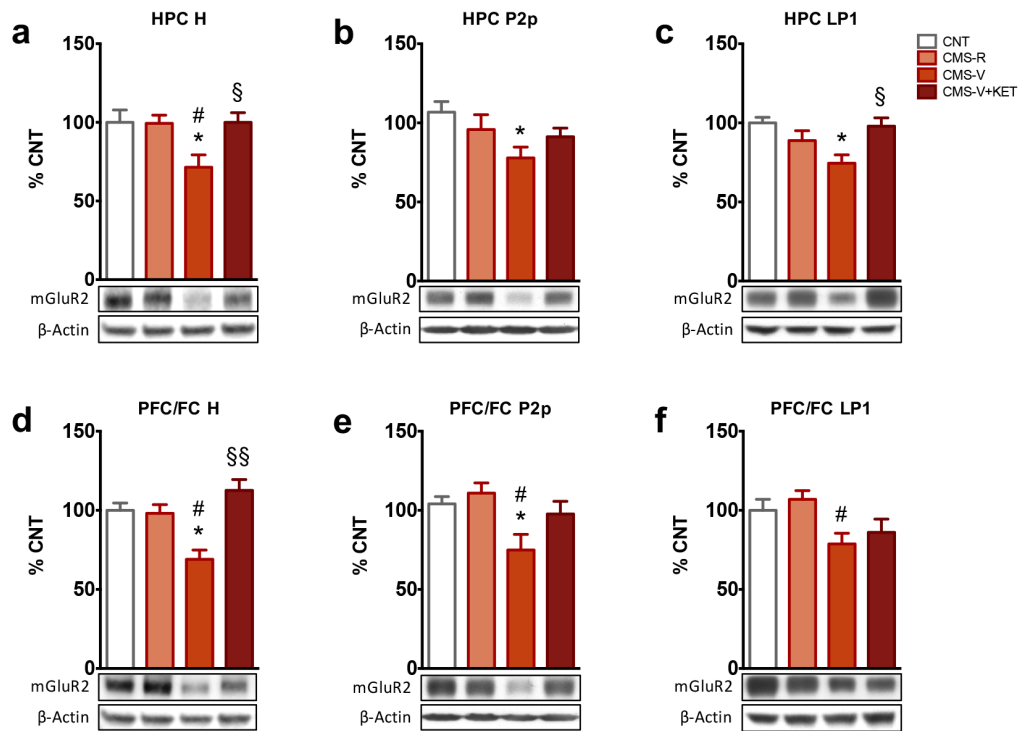
Figure 4.10: mGluR2 protein levels

Expression of mGluR2 protein on **(a)** homogenate, **(b)** synaptosomes and **(c)** synaptic membranes from HPC. Expression of mGluR2 protein on **(d)** homogenate, **(e)** synaptosomes and **(f)** synaptic membranes from PFC/FC. n = 10-12. *$p<0.05$ vs CNT; [#]$p<0.05$ vs CMS-R; [§]$p<0.05$ vs CMS-V; [§§]$p<0.001$ vs CMS-V.

4.5 Effect of CMS and KET on cellular/synaptic pathways

We then evaluated the effect of CMS and KET on the total expression and phosphorylation levels (as an index of activation) of two critical regulators of cellular and synaptic functions: the mammalian target of rapamycin (mTOR) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).

4.5.1 No effect of CMS and KET on mTOR expression and activity

mTOR is a kinase involved in numerous fundamental cellular and neuronal functions, including cell survival, energy balance, protein synthesis, circuit formation and synaptic plasticity (*Lipton and Sahin, 2014*). Importantly, KET rapid antidepressant effect was shown to rely on mTOR pathway (*Li et al., 2010; Li et al, 2011*)

mTOR total levels were not affected by KET and CMS in HPC homogenate ($F_{3,20} = 0.87$, $p = 0.4724$, one-way ANOVA) and synaptosomes ($F_{3,22} = 0.91$, $p = 0.4522$, one-way ANOVA), nor in PFC/FC homogenate ($F_{3,20} = 0.21$, $p = 0.8858$, one-way ANOVA) and synaptosomes ($F_{3,23} = 1.19$, $p = 0.3368$, one-way ANOVA) (*Fig. 4.11*).
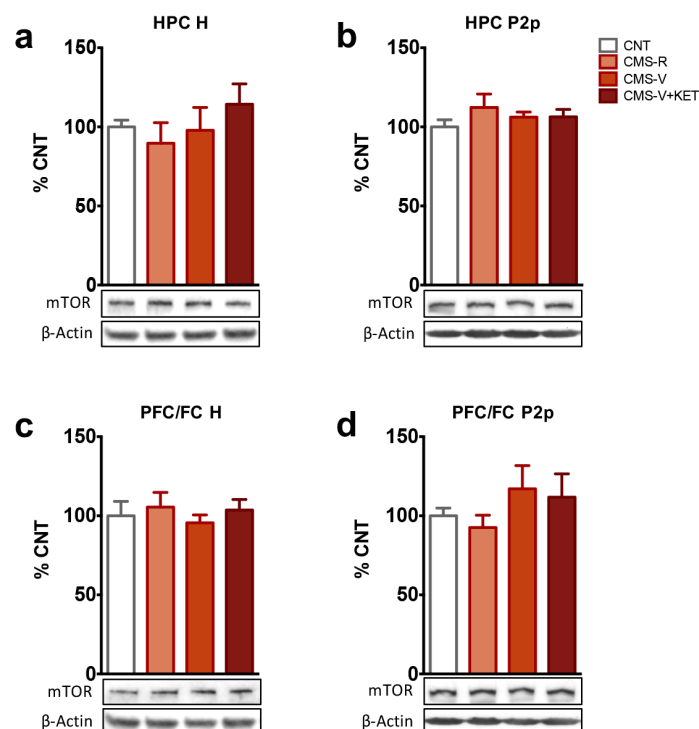
Figure 4.11: mTOR protein levels

Expression of mTOR protein on **(a)** homogenate and **(b)** synaptosomes from HPC. Expression of mTOR protein on **(c)** homogenate and **(d)** synaptosomes from PFC/FC. n = 8-10.

We did not even found effects of CMS and KET on the phosphorylation of mTOR at Ser^{2448} in HPC homogenate ($F_{3,20}$ = 0.58, p = 0.6321, one-way ANOVA) and synaptosomes ($F_{3,21}$ = 0.97, p = 0.4263, one-way ANOVA), or in PFC/FC homogenate ($F_{3,20}$ = 0.61, p = 0.6190, one-way ANOVA) and synaptosomes ($F_{3,23}$ = 1.15, p = 0.3489, one-way ANOVA) (*Fig. 4.12*).
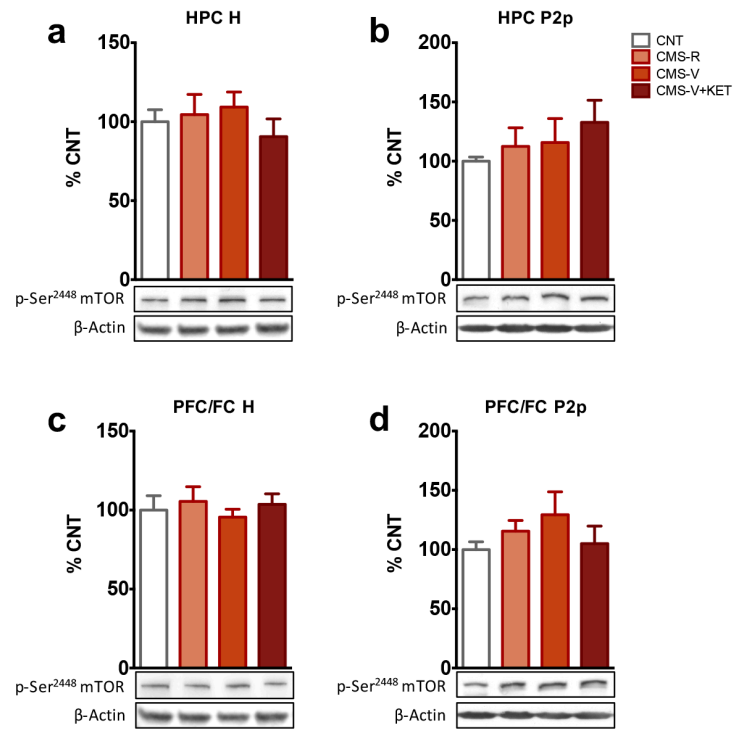
Figure 4.12: phospho-Ser^{2448} mTOR protein levels

Expression of phospho-Ser^{2448} mTOR protein on **(a)** homogenate and **(b)** synaptosomes from HPC. Expression of phospho-Ser^{2448} mTOR on **(c)** homogenate and **(d)** synaptosomes from PFC/FC. n = 8-10.

4.5.2 Phosphorylation of CaMKII was decreased in vulnerable animals and restored by KET

CaMKII is a synaptic protein, particularly abundant at glutamatergic synapses, where is involved at both pre- and post-synaptic levels in the regulation of key synaptic functions, such as neurotransmitter synthesis and release, calcium homeostasis or synaptic plasticity (*Lisman et al., 2002; Wang, 2008*).

We found no changes in the total levels of CaMKII in HPC homogenate ($F_{3,44}$ = 0.04, p = 0.9897, one-way ANOVA), synaptosomes ($F_{3,21}$ = 2.16, p = 0.1224, one-way ANOVA) and synaptic membranes ($F_{3,20}$ = 3.25, p = 0.7667, one-way ANOVA), as well as in PFC/FC homogenate ($F_{3,25}$ = 0.89, p = 0.4556, one-way ANOVA), synaptosomes ($F_{3,20}$ = 0.38, p = 0.7703, one-way ANOVA) and synaptic membranes ($F_{3,20}$ = 1.25, p = 0.3180, one-way ANOVA) (*Fig. 4.13*).
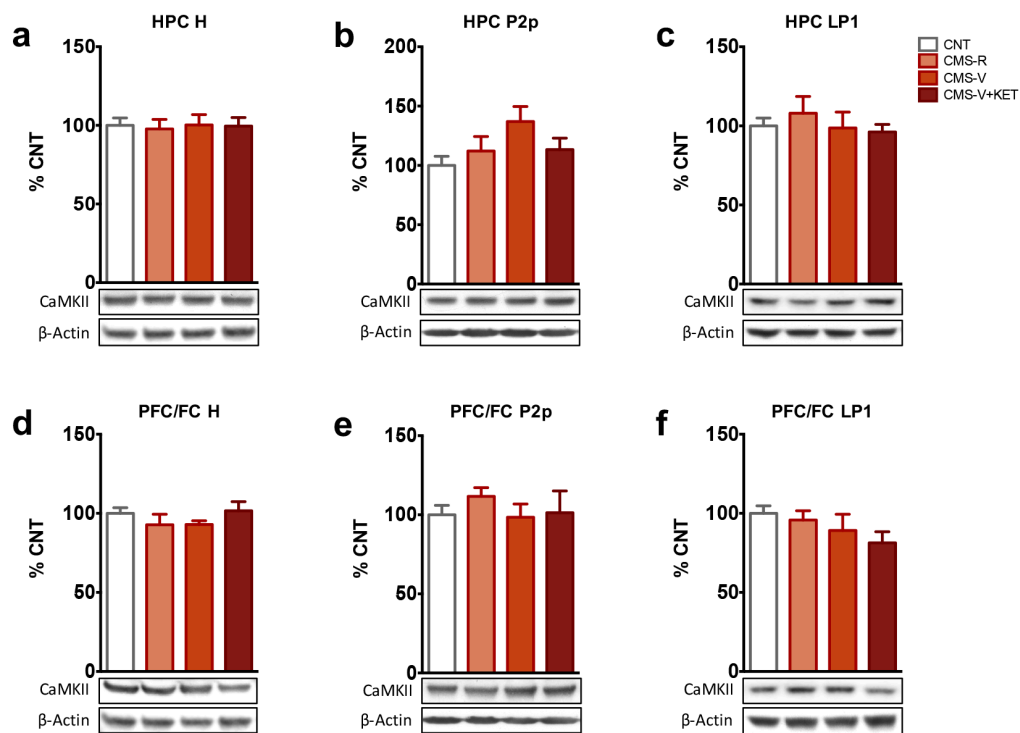
Figure 4.13: CaMKII protein levels

Expression of CaMKII protein on **(a)** homogenate, **(b)** synaptosomes and **(c)** synaptic membranes from HPC. Expression of CaMKII protein on **(d)** homogenate, **(e)** synaptosomes and **(f)** synaptic membranes from PFC/FC. n = 8-12.

A significant decrease of phospho-Thr[286] CaMKII was instead found in homogenate of CMS-V HPC; KET restored CaMKII phosphorylation to control levels ($F_{3,63}$ = 3.78, p<0.05, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT p<0.05; CMS-V vs.

CMS-R $p<0.05$; CMS-V+KET vs. CMS-V $p<0.05$) (*Fig. 4.13-a*). No effects of CMS and KET were found in synaptosomes ($F_{3,31} = 1.85$, $p = 0.1581$, one-way ANOVA) and synaptic membranes ($F_{3,31} = 1.06$, $p = 0.3922$, one-way ANOVA) (*Fig. 4.13-b/c*).

As for the HPC, also in the PFC/FC homogenate CMS-V induced a significant decrease of phospho-Thr^{286} CaMKII levels, which was completely rescued by KET treatment ($F_{3,42} = 4.05$, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT $p<0.05$; CMS-V vs. CMS-R $p<0.05$; CMS-V+KET vs. CMS-V $p<0.05$) (*Fig. 4.13-d*). No changes of CaMKII phosphorylation were found in PFC/FC synaptosomes ($F_{3,30} = 0.70$, $p = 0.5553$, one-way ANOVA) and in synaptic membranes ($F_{3,20} = 0.26$, $p = 0.8552$, one way ANOVA) (*Fig. 4.13-e/f*).
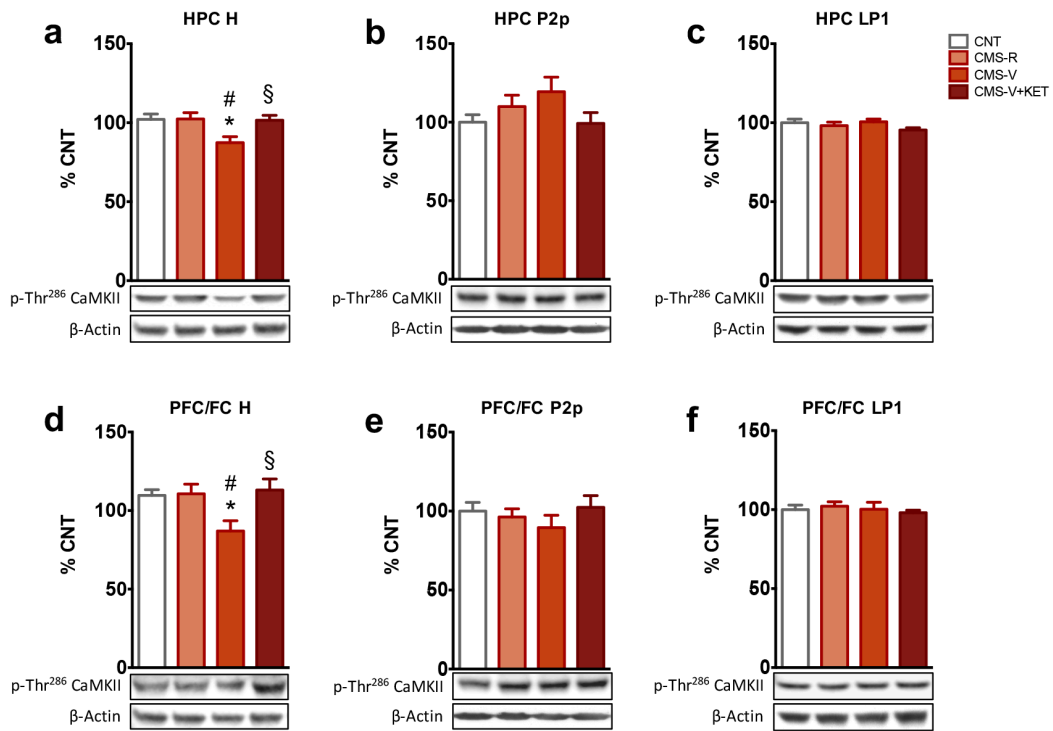
Figure 4.14: phospho-Thr^{286} CaMKII protein levels

Expression of phospho-Thr^{286} CaMKII protein on **(a)** homogenate, **(b)** synaptosomes and **(c)** synaptic membranes from HPC. Expression of phospho-Thr^{286} CaMKII protein on **(d)** homogenate, **(e)** synaptosomes and **(f)** synaptic membranes from PFC/FC. n = 8-12. *$p<0.05$ vs CNT; #$p<0.05$ vs CMS-R; §$p<0.05$ vs CMS-V.

4.6 Effect of CMS and KET on BDNF mRNA

4.6.1 CMS reduced total BDNF transcript in HPC and PFC/FC

Since several studies highlighted the central role of BDNF in both stress-related disorders and antidepressant treatments (*Castrén, 2004*), we analyzed the mRNA levels of total BDNF mRNA by qPCR. The overall mRNA levels in the HPC were decreased by CMS in both CMS-V and CMS-R ($F_{3,34} = 8.72$, $p<0.001$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.05$; CMS-V vs. CNT $p<0.001$) (*Fig. 4.15-a*); KET treatment had no effect on total BDNF mRNA levels (CMS-V+KET vs. CNT $p<0.001$). Similar results were obtained in PFC/FC, where reduced total BDNF mRNA levels were found in CMS-R, CMS-V and CMS-V+KET ($F_{3,32} = 4.72$, $p<0.01$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.05$; CMS-V vs. CNT $p<0.001$; CMS-V+KET vs. CNT p<0.001) (*Fig. 4.15-b*).
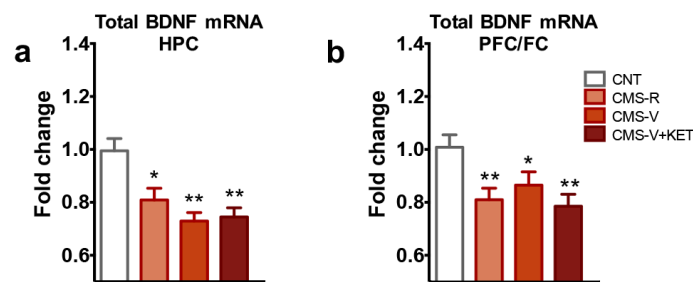
Figure 4.15: Total BDNF mRNA levels

Total BDNF mRNA levels on **(a)** HPC and **(b)** PFC/FC homogenate. n = 9-10. *p<0.05 vs CNT; *p<0.01 vs CNT.

4.6.2 CMS induced brain-area specific changes in BDNF splice variants mRNAs

As transcription of BDNF is regulated by at least 9 different promoters, each giving rise to a different splice variant transcript (*Sakata et al., 2009*), we also analyzed the effect of CMS and KET on the transcription of the BDNF splice variants mostly expressed in the rat brain: BDNF-1, BNDF-2, BDNF-4 and BDNF-6 transcripts.

We found that CMS caused a general reduction of the expression of BDNF transcripts in all animals. BDNF-1 mRNA levels are significantly reduced in the HPC of CMS-V and CMS-V+KET, while in CMS-R animals the reduction did not reach the significance ($F_{3,34} = 4.97$, $p<0.01$, one-way ANOVA; Tukey's post hoc test: CMS-V vs. CNT p<0.01; CMS-V+KET vs. CNT p<0.05) (*Fig. 4.16-a*). BDNF-2 mRNA levels were significantly

reduced in HPC of CMS-R, CMS-V, and CMS-V+KET ($F_{3,28}$ = 7.89, $p<0.001$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.001$; CMS-V vs. CNT $p<0.01$; CMS-V+KET vs. CNT $p<0.05$) (*Fig. 4.16-b*), as well as BDNF-4 mRNA levels ($F_{3,34}$ = 13.85, $p<0.001$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.001$; CMS-V vs. CNT $p<0.001$; CMS-V+KET vs. CNT $p<0.001$) (*Fig. 4.16-c*), and BDNF-6 levels ($F_{3,44}$ = 5.74, $p<0.01$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.05$; CMS-V vs. CNT $p<0.01$; CMS-V+KET vs. CNT $p<0.01$) (*Fig. 4.16-d*).
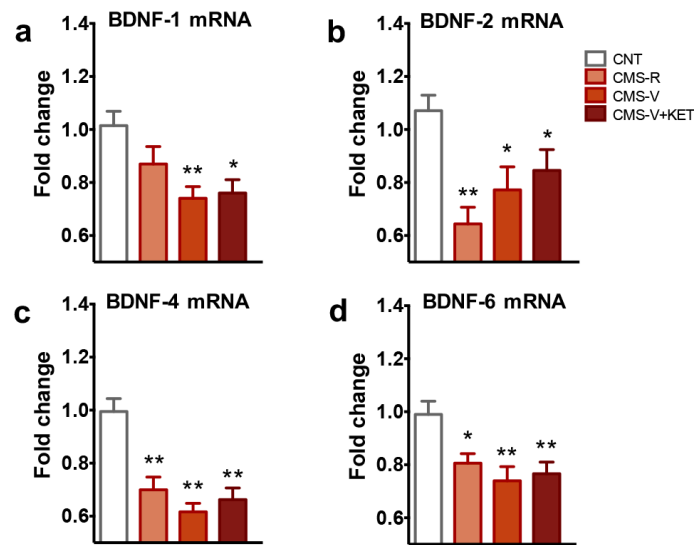
Figure 4.16: BDNF splice variants mRNA levels in HPC

(a) BDNF-1, **(b)** BDNF-2, **(c)** BDNF-4 and **(d)** BDNF-6 mRNA levels in HPC homogenate. n = 9-12. *p<0.05 vs CNT; *p<0.01 vs CNT.

On the other hand, in PFC/FC BDNF-1 levels were significantly reduced only in CMS-R ($F_{3,30}$ = 4.36, $p<0.05$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.01$) (*Fig. 4.17-a*). BDNF-2 mRNA levels were significantly reduced in PFC/FC of CMS-R and CMS-V+KET, while a trend to decrease was found in CMS-V ($F_{3,31}$ = 5.14, $p<0.01$, one-way ANOVA; Tukey's *post hoc* test: CMS-R vs. CNT $p<0.01$; CMS-V+KET vs. CNT $p<0.05$; CMS-V vs. CNT p = 0.2573; CMS-V vs. CMS-R p = 0.2004) (*Fig. 4.17-b*). Finally, we found no difference between experimental groups in the levels of BDNF-4 ($F_{3,32}$ = 2.28, p = 0.0981, one-way ANOVA) and BDNF-6 ($F_{3,32}$ = 0.87, p = 0.4657, one-way ANOVA) transcripts in PFC/FC (*Fig. 4.17-c/d*).
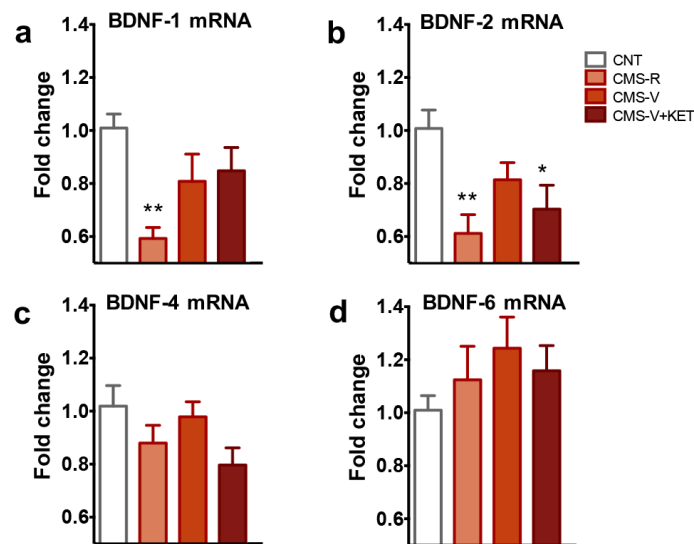
Figure 4.17: BDNF splice variants mRNA levels in PFC/FC

(a) BDNF-1, **(b)** BDNF-2, **(c)** BDNF-4 and **(d)** BDNF-6 mRNA levels in PFC/FC homogenate. n = 9-12. *p<0.05 vs CNT; *p<0.01 vs CNT.

4.6.3 Dendritic trafficking of total BDNF and BDNF splice variants transcripts was altered by CMS and modulated by KET

We previously found that chronic treatment with classical antidepressants, as well as physical exercise, are able to increase the trafficking of BDNF mRNA to the dendrites (*Baj et al., 2012*). This effect was suggested to promote local translation of BDNF and consequent dendritic remodeling. Here we performed an *in-situ* hybridization analysis to understand the effects of CMS and KET on dendritic targeting of total BDNF, BDNF-2 and BDNF-6 transcripts, in CA1 and CA3 regions of HPC and in PFC/FC.

A significant decrease in the trafficking of total BDNF mRNA was found in CA1 of both CMS-R and CMS-V; this reduction was higher in CMS-V compared to CMS-R and restored to the levels of CMS-R by KET treatment (Multilevel Covariance Analysis; CMS-R vs. CNT $p<0.001$; CMS-V vs. CNT $p<0.001$; CMS-V vs. CMS-R $p<0.001$; CMS-V+KET vs. CNT $p<0.001$; CMS-V+KET vs. CMS-V $p <0.001$) (*Fig. 4.18-a*). Instead, in CA3, we found a significant decrease in the trafficking of total BDNF mRNA selectively in CMS-V; interestingly, this effect was completely restored by KET (Multilevel Covariance Analysis; CMS-V vs. CNT $p<0.001$; CMS-V vs. CMS-R $p<0.001$; CMS-V+KET vs. CMS-V $p <0.001$) (*Fig. 4.18-b*). Conversely, no changes were observed

for total BDNF in PFC/FC (Multilevel Covariance Analysis) mRNA trafficking (*Fig. 4.18-c*).
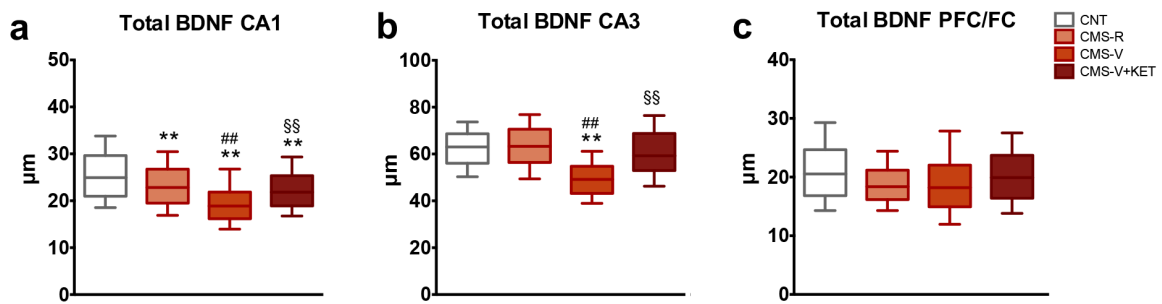
Figure 4.18: Dendritic trafficking of BDNF splice variants mRNA in HPC and PFC/FC
Dendritic trafficking (μm) of total BDNF mRNA in **(a)** CA1 and **(b)** CA3 of HPC, and in **(c)** layer V of PFC/FC. n = 6-10. **p<0.001 vs CNT; ##p<0.001 vs CMS-R; §§p<0.001 vs CMS-V.

We also analyzed changes in dendritic trafficking of BDNF-2 and BDNF-6 splice variants transcripts, the main BDNF transcripts translocated to distal dendrites (*Baj et al., 2011*).

In the CA1 region of HPC, BDNF-2 dendritic trafficking was increased in CMS-R, while decreased in CMS-V rats; KET treatment completely restored this change; (Multilevel Covariance Analysis; CMS-R vs. CNT *p*<0.001; CMS-V vs. CNT *p*<0.001; CMS-V vs. CMS-R *p*<0.001; CMS-V+KET vs. CMS-V *p* <0.001) (*Fig. 4.19-a*). Similar results were also obtained in the CA3: BDNF-2 dendritic trafficking was increased in CMS-R, reduced in CMS-V and restored to CNT levels by acute KET (Multilevel Covariance Analysis; CMS-R vs. CNT *p*<0.001; CMS-V vs. CNT *p*<0.001; CMS-V vs. CMS-R *p*<0.001; CMS-V+KET vs. CMS-V *p* <0.001) (*Fig. 4.19-b*). Due to the low expression levels of BDNF-2 transcript in PFC/FC, we were not able to detect its signal with *in situ* hybridization in this area.

Dendritic trafficking of BDNF-6 also was oppositely regulated in the CA1 of CMS-R and CMS-V animals: while BDNF-6 dendritic translocation was increased in CMS-R, it was reduced in CMS-V; no effect of KET was detected (Multilevel Covariance Analysis; CMS-R vs. CNT *p*<0.001; CMS-V vs. CNT *p*<0.001; CMS-V vs. CMS-R *p*<0.001; CMS-V+KET vs. CMS-R *p*<0.001; CMS-V+KET vs. CMS-V *p* <0.001) (*Fig. 4.19-c*). In CA3, we found a reduction of BDNF-6 dendritic trafficking selectively in CMS-V; KET was able to fully revert this change (Multilevel Covariance Analysis; CMS-V vs. CNT *p*<0.001; CMS-V vs. CMS-R *p*<0.001; CMS-V+KET vs. CMS-V *p* <0.001) (*Fig. 4.19-d*).

In layer V of PFC/FC, a significant increase of BDNF-6 dendritic trafficking was found in CMS-R and CMS-V+KET, compared to both CNT and CMS-V (Multilevel Covariance Analysis; CMS-R vs. CNT $p<0.001$; CMS-V vs. CMS-R $p<0.001$; CMS-V+KET vs. CNT $p<0.001$; CMS-V+KET vs. CMS-R $p<0.001$; CMS-V+KET vs. CMS-V $p<0.001$) (*Fig. 4.19-e*).
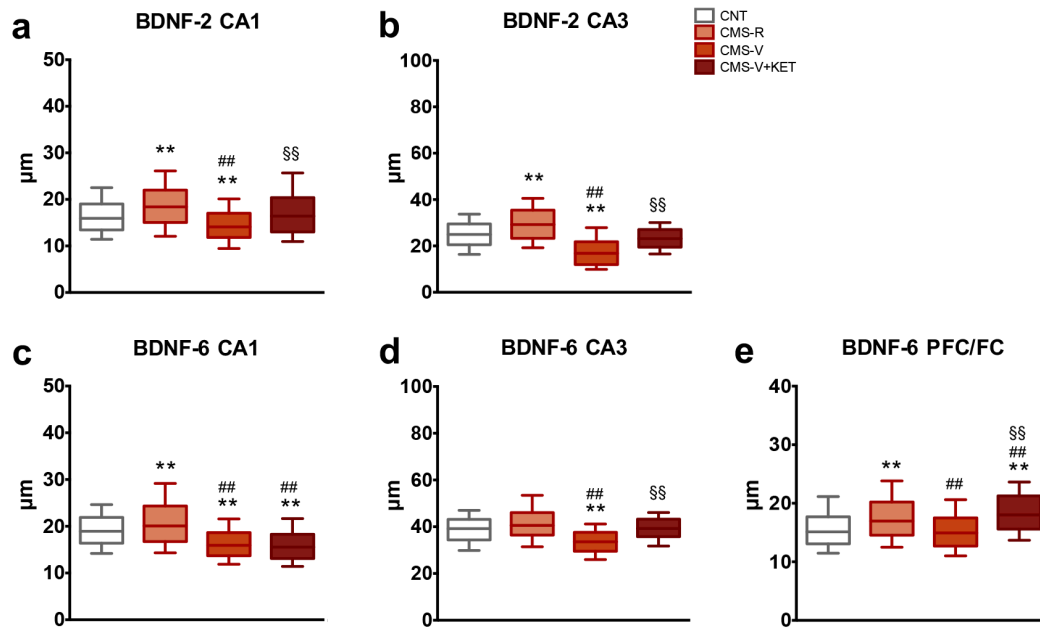
Figure 4.19: Dendritic trafficking of total BDNF mRNA in HPC and PFC/FC

Dendritic trafficking (μm) of BDNF-2 mRNA in **(a)** CA1 and **(b)** CA3 of HPC. Dendritic trafficking (μm) of BDNF-6 mRNA in **(c)** CA1 and **(d)** CA3 of HPC, and in layer V of PFC/FC. n = 6-10. **p<0.001 vs CNT; ##p<0.001 vs CMS-R; §§p<0.001 vs CMS-V.

4.7 Effect of CMS and KET on dendritic morphology

Due to the central role of BDNF in the regulation of structural plasticity and synaptogenesis (*Castrén, 2004*), we then studied whether the altered trafficking of BDNF mRNA observed in CA3 could correlate with changes in dendritic morphology in the same area. CA3 pyramidal neurons were analyzed by subdividing apical from basal dendrites (*Nava et al., 2015; Chen F. et al., 2016*).

4.7.1 CMS induced retraction of CA3 apical dendrites in vulnerable rats. KET restored these changes

One-way ANOVA followed by Tukey's *post hoc* test revealed that CMS significantly reduced the total length of apical dendrites in the CA3 of CMS-V ($F_{3,38}$ = 5.67, $p<0.01$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT $p<0.05$; CMS-V vs. CMS-R $p<0.05$) (*Fig. 4.20-a*). Intriguingly, this effect was completely restored to control levels by KET treatment (CMS-V+KET vs. CMS-V $p<0.01$). Accordingly, the same effects of CMS and KET were found in the total number of branches of CA3 apical dendrites ($F_{3,38}$ = 5.52, $p<0.01$, one-way ANOVA; Tukey's *post hoc* test: CMS-V vs. CNT $p<0.05$; CMS-V vs. CMS-R p = 0.0678; CMS-V+KET vs. CMS-V $p<0.01$) (*Fig. 4.20-b*).

Conversely, we found no significant difference between experimental groups in the total length ($F_{3,38}$ = 0.47, p = 0.7011, one-way ANOVA) and branching number ($F_{3,38}$ = 0.61, p = 0.6130, one-way ANOVA) of basal dendrites (*Fig. 4.20-c/d*).

Two-way ANOVA of Sholl analysis revealed a significant effect of CMS ($F_{3,90}$=10.89, $p<0.001$) and distance ($F_{30,90}$=117.5, $p<0.001$) on the number of intersections of apical dendrites with the concentric 20 μm-Sholl radii. In particular, Tukey's *post hoc* test showed that CMS significantly decreased the number of intersections of distal apical dendrites (between 300 μm and 380 μm) selectively in the CA3 of CMS-V (CMS-V vs. CNT $p<0.05$; CMS-V vs. CMS-R $p<0.05$); KET rescued this effect in CMS-V (CMS-V+KET vs. CMS-V $p<0.05$;) (*Fig. 4.21-a*). No significant effect of CMS was found in basal dendrites ($F_{3,39}$ = 1.25, p = 0.3033, two-way ANOVA) (*Fig. 4.21-b*).
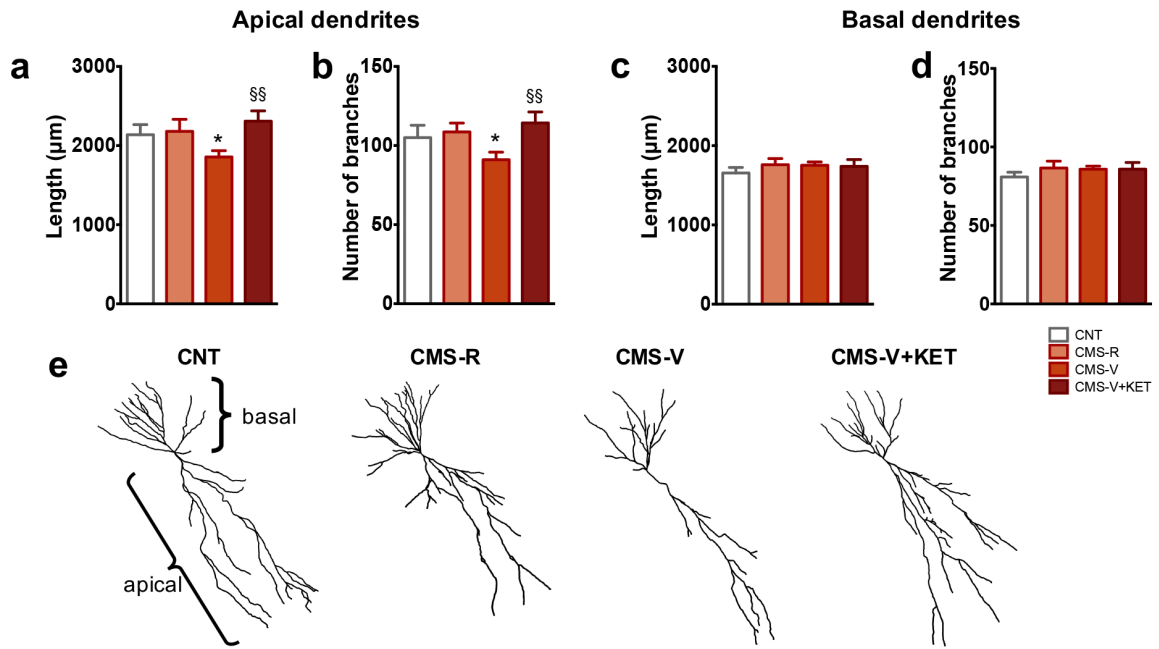
Figure 4.20: Dendritic morphology of CA3 pyramidal neurons

(a) Total length and (b) branching number of apical dendrites. (c) Total length and (d) branching number of basal dendrites. (e) Representative drawings of CA3 pyramidal neurons reconstructed with Imaris software. n = 10-12. *$p<0.05$ vs CNT; §§$p<0.001$ vs CMS-V.
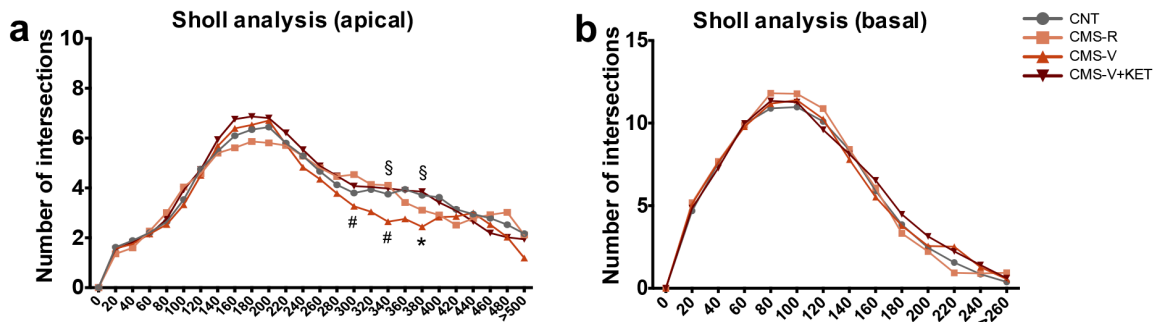
Figure 4.21: Sholl analysis of CA3 pyramidal neurons

Sholl analysis of (a) apical and (b) basal dendrites. n = 10-12. *$p<0.05$ vs CNT; #$p<0.05$ vs CMS-R; §$p<0.05$ vs CMS-V.

4.7.2 No effect of CMS and KET on dendritic spines

No significant difference in the density of dendritic spines was found in apical dendrites ($F_{3,34}$ = 0.30, p = 0.8194, one-way ANOVA) (*Fig. 4.22-a*). Moreover, we found no effect of CMS and KET on the density of stubby ($F_{3,34}$ = 1.55, p = 0.2205, one-way ANOVA), mushroom ($F_{3,34}$ = 0.29, p = 0.8333, one-way ANOVA), filopodia ($F_{3,34}$ = 0.86, p = 0.4711, one-way ANOVA) and long thin ($F_{3,34}$ = 0.17, p = 0.9166, one-way ANOVA) spine subtypes (*Fig. 4.22-b*).
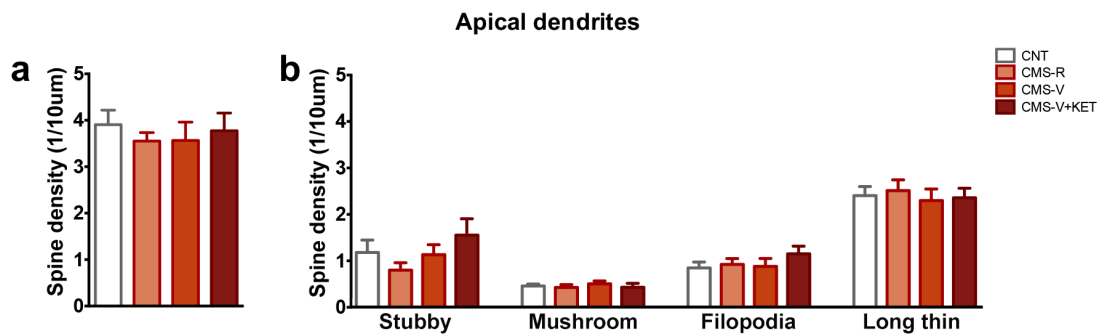
Figure 4.22: Spine density of apical dendrites from CA3 pyramidal neurons

(a) Density of the total number of spines on apical dendrites. **(b)** Density of each spine subtype (stubby, mushroom, filopodia, long thin) on apical dendrites. n = 10-12.

5. DISCUSSION

5.1 KET rescues anhedonic behavior in CMS-V

CMS is a well-validated and widely used animal model of depression, that induces many symptomatic parallels to MDD, including behavioral and neurobiological alterations (*Willner, 1997; Hill et al., 2012*). Among these, the primary behavioral outcome of CMS is anhedonia, a core symptom of depression, that consists in decreased responsiveness to rewarding stimuli (*Nestler et al., 2002*).

In the present work, we set-up the CMS model of depression, by exposing rats to a variety of mild and unpredictable stressors, over a period of 5 weeks (*Willner et al., 1987; Banasr et al., 2007; Li et al., 2011*). Since anhedonia is usually assessed in rodents by measuring their preference for a palatable sweet solution over water (*Nestler and Hyman, 2010; Willner, 2017a*), we validated the CMS paradigm by monitoring hedonic behavior of rats with SPT.

In line with previously published studies, repeatedly showing the anhedonic effect of CMS (*Willner et al., 1987; Garcia et al., 2009; Li et al., 2011; Willner, 2017a*), we found that rats in the CMS group displayed an overall decrease in the preference for sucrose. However, although all the animals were exposed to the same stress paradigm for an equal amount of time, only approximately half of the rats subjected to CMS actually displayed an anhedonic behavior, while the remaining rats maintained their baseline preference for sucrose. This observation is in line with increasing evidence in literature, reporting a remarkable inter-individual variability in hedonic behavior after exposure to stress paradigms (*Willner, 2005; Krishnan and Nestler, 2011; Strekalova et al., 2011*). Since distinguishing vulnerable and resilient animals may allow to better characterize the mechanisms underlying individual response to stress (*Strekalova and Steinbusch, 2010;*

Strekalova et al., 2011; Wiborg, 2013), we separated rats that were vulnerable to CMS from resilient ones by applying a cut-off at 55% of sucrose preference. Rats showing a preference for sucrose higher than 55% were classified as CMS-R, while anhedonic rats, showing a preference lower than 55%, were classified as CMS-V.

Several studies also showed that the anhedonic behavior induced by CMS may be reversed by chronic treatment with conventional antidepressants (*Willner et al. 1987; Papp et al. 1996; Larsen et al., 2010; Zhang et al., 2010*), as well as by an acute treatment with KET (*Li et al., 2011; Choi et al., 2015; Sun et al., 2016; Hare et al., 2017; Papp et al., 2017*). However, in most of these studies antidepressant drugs have been administered without discriminating for individual susceptibility to stress. In the present work, we investigated the nature of the fast antidepressant action of KET, by selectively treating animals that displayed a depressive-like phenotype. At the fifth week of CMS, half of the CMS-V rats were administered with a single sub-anesthetic dose of KET, and SPT was performed 24 hours after KET treatment. Our results show that acute KET completely rescued hedonic behavior in CMS-V, confirming its fast antidepressant effect.

5.2 CMS induces major phenotypic changes in CMS-V

We also validated the CMS paradigm by measuring different phenotypic changes induced by stress. Overall, we found that anhedonic rats displayed more marked phenotypic changes than CMS-R.

First, body weight gain was monitored twice a week in both CNT and CMS rats. Indeed, studies in literature have demonstrated that repeated exposure to stressful situations can influence feeding behavior and cause reduction in body weight gain (*Bekris et al., 2005; Garcia et al., 2009*; *Henningsen et al, 2012; Chen J. et al, 2016*). Similarly, depressed patients often show altered appetite, resulting in significant weight loss (*American Psychiatric Association, 2013*). In agreement with literature, our findings show that rats subjected to CMS have a significant loss of body weight gain compared to non-stressed rats. Although this effect was present in both CMS-R and CMS-V, the reduction in body weight was significantly more evident in CMS-V compared to CMS-R.

Since the HPA axis is a key mediator of the stress response (*Nestler et al., 2002*), we also measured the adrenal glands/total body weight ratio and CORT serum levels. Several studies already reported hypertrophy of adrenal glands as a consequence of stress-induced HPA hyperactivity, both in rodents (*Herman et al., 1995; Pignatelli et al., 2000; Garcia et al., 2009; Cai et al., 2015*), and in depressed patients (*Swaab et al., 2005; Lucassen et al., 2014*). Accordingly, we found that CMS increased the weight of adrenal glands, although the significance was reached only for CMS-V animals.

In line with previous studies consistently reporting that stress increases the serum levels of CORT (*Garcia et al., 2009; Réus et al., 2012; Chen J. et al, 2016*), in our study all the rats subjected to CMS showed increased CORT levels; however, we found higher CORT levels in CMS-V than in CMS-R rats. This robust surge in serum CORT levels measured in CMS-V, paralleled by the increased weight of their adrenal glands, suggest that abnormal hyperactivity of the HPA axis may be involved in the onset of the depressive-like behavior exhibited by these animals.

Intriguingly, 24 hours after an acute treatment with KET, we found no effect of KET on HPA alterations induced by CMS in CMS-V, suggesting that KET may exert its rapid antidepressant effect, acting downstream of CORT pathway.

5.3 CMS differently affects excitatory presynaptic release in CMS-V and CMS-R

Converging lines of evidence have shown that acute stress rapidly increases excitatory neurotransmission in HPC and PFC/FC (*Lowy et al., 1995; Yuen et al., 2009*). However, the effect of chronic stress on glutamate transmission is still elusive. Microdialysis studies reported that repeated stressors in rats produce transient glutamate effluxes in HPC and PFC/FC (*Moghaddam, 2002; Yamamoto and Reagan, 2006*), while tissue analysis performed 24 hours following the conclusion of CMS paradigm, found increased glutamate concentrations in the same brain regions (*Garcia-Garcia et al., 2009*).

It has also been observed that KET exerts its antidepressant effect by inducing a transient burst of glutamate release (*Moghaddam et al., 1997*), although this effect seems to be

apparently in contrast with the enhancement of glutamate release and transmission seen following stress.

So far, the effects of chronic stress and KET on glutamate transmission have mostly been studied by using techniques (such as microdialysis) that cannot give a true account of presynaptic release, since they only detect total extracellular glutamate (heavily contaminated by metabolic glutamate), of which presynaptically released glutamate is a minor part (*Popoli et al, 2012*). Thus, in the present work, we measured glutamate presynaptic release by using the superfusion of purified synaptosomes, a technique that allows to precisely measure both the basal and depolarization-evoked presynaptic releases of endogenous neurotransmitters (*Raiteri and Raiteri, 2000; Bonanno et al., 2005; Treccani et al., 2014; Musazzi et al., 2017*).

5.3.1 CMS impairs excitatory neurotransmitters release in HPC of CMS-V

Our results show that CMS reduces both basal and depolarization-evoked releases of glutamate selectively in HPC from CMS-V. Although this observation seems apparently in contrast with the elevated glutamate levels reported in HPC tissues following CMS (*Garcia-Garcia et al., 2009*), we can speculate that a reduction in glutamate presynaptic release may represent a compensatory mechanism limiting a further increase of extrasynaptic glutamate levels. A possible evidence for this hypothesis comes from a previous study in literature, showing that CMS reduces the expression of the vesicular glutamate transporter-1, responsible for the transport of glutamate into synaptic vesicles (*Elizalde et al., 2010*). However, further studies on the mechanisms underlying CMS induced reduction of glutamate release in the HPC of CMS-V are needed to test this hypothesis.

Previous evidence in literature has reported that basal release of glutamate activates specific intracellular signaling cascades involved in the modulation of action potential firing, maturation and stability of synaptic networks, local dendritic protein synthesis, and homeostatic synaptic plasticity (*Kavalali et al., 2011; Nosyreva et al., 2013*). Therefore, although the mechanisms underlying the reduction of basal glutamate release in CMS-V are not clear, this evidence may suggest that CMS could alter synaptic homeostasis in vulnerable subjects.

In this context, it is important to notice that KET was suggested to exert its antidepressant effect by specifically acting on NMDA receptors that modulate the response to spontaneously released glutamate (*Kavalali and Monteggia, 2015; Monteggia and Zarate, 2015*). Intriguingly, our findings show that basal, but not evoked, release of glutamate was not reduced in HPC synaptosomes from CMS-V rats treated with KET, suggesting that KET may be at least in part able to rescue spontaneous transmission and synaptic homeostasis in vulnerable animals.

Previous studies have shown that KET may impact on depolarization-evoked glutamate neurotransmission; however, this was reported using higher doses of KET, and was associated with the triggering of psychotomimetic consequences of the drug (*Kavalali and Monteggia, 2012*). The absence of KET effect on depolarization-evoked release in our model may be explained by the low, sub-anesthetic, doses employed in this study. Importantly, it has been recently proposed that drugs that selectively target spontaneous neurotransmitter release, without affecting evoked neurotransmission, may represent a promising therapeutic approach to MDD (*Kavalali and Monteggia, 2012*).

We also assessed the effects of CMS and KET on presynaptic release of aspartate, another excitatory neurotransmitter in the brain. Similarly to glutamate, reductions in basal and depolarization-evoked release of aspartate were found in CMS-V, although reduced basal aspartate release was also observed in CMS-R. Intriguingly, KET completely rescued basal, but not evoked, release of aspartate in CMS-V. The biological and functional consequences of these changes, although still unknown, added with the alterations of glutamate transmission, could impact on excitatory transmission in the HPC.

Overall, these results suggest that CMS impairs basal and evoked releases of excitatory neurotransmitters in the HPC of CMS-V, and that KET is able to reverse changes in basal release.

5.3.2 CMS reduces glutamate release in PFC/FC of CMS-R

Surprisingly, our findings show opposite changes in PFC/FC from CMS-R and CMS-V animals. Indeed, while in HPC we reported a reduction of basal and depolarization evoked release of glutamate in CMS-V, with no changes in CMS-R, both basal and depolarization evoked release of glutamate were significantly reduced in the PFC/FC of CMS-R rats, and not in CMS-V. Moreover, we found no significant effect of KET on glutamate release. No

changes were measured in the presynaptic release of aspartate. Future studies will help in clarifying whether the reduction of glutamate release seen in PFC/FC of CMS-R may represent a pro-adaptive or a maladaptive change in response to CMS.

5.4 CMS differently affects basal and evoked presynaptic GABA release in HPC

By using the superfusion of purified synaptosomes, we also assessed the effect of CMS and KET on inhibitory transmission, by measuring basal and depolarization-evoked presynaptic release of GABA.

Our results show that CMS differently affected basal and evoked releases of GABA in HPC of CMS-R and CMS-V. In particular, we found an increase in basal release of GABA in CMS-R. Although increased basal GABA release would suggest an increased inhibitory tone, this change does not seem to be consistent with glutamate basal and evoked releases observed in the HPC of CMS-R.

Conversely, GABA depolarization-evoked release was decreased in HPC from CMS-V. This evidence is consistent with previous studies in literature showing reductions of GABA levels in HPC of rodents following CMS (*Grønli et al., 2007; Garcia-Garcia et al., 2009; Elizalde et al., 2010*), as well as in the brain of MDD patients (*Lusher et al., 2011*).

Interestingly, this reduction was completely rescued by KET treatment in CMS-V. This observation seems to be in contrast with the hypothesis that KET induces a transient burst of glutamate release, by inhibiting GABAergic interneurons (*Abdallah et al., 2015; Duman et al., 2016*). However, it is worth noting that KET only exerts a fast and transient inhibitory effect on GABAergic interneurons (*Homayoun and Moghaddam, 2007*), and KET itself has a relatively short half-life (*Kavalali and Monteggia, 2015*). Thus, the effect we observed on HPC synaptosomes 24 hours after KET treatment may not necessarily exclude a possible initial inhibitory action of KET on GABA transmission.

Finally, our results show no effect of CMS and KET on GABA presynaptic release in PFC/FC, once again suggesting that CMS differently modulated presynaptic neurotransmitter release between PFC/FC and HPC.

5.5 KET modulates the response of MR and GR to CORT in CMS-V

5.5.1 CMS increases the synaptic localization of MR

MR is a high affinity receptor for glucocorticoids, and the CORT receptor mostly involved in the regulation of neuronal activity and stress response in the CNS (*Joëls et al., 2012*). MR is expressed at synapses and, once activated, can be translocated to the nucleus, where it regulates gene transcription (*Joëls et al., 2017*).

Several studies have previously reported that CMS downregulates the expression of MR in HPC (*Kim et al., 1999; Hill et al., 2012*), although increased expression in the dentate gyrus was also reported (*Van Riel et al., 2003*). Conversely, no effect of CMS has been reported for PFC/FC (*Kim et al., 1999; Hill et al., 2012*).

Our results show no difference between experimental groups in MR protein levels in HPC and PFC/FC homogenate, suggesting that the overall expression of MR is not affected by CMS. However, we found increased MR levels in HPC and PFC/FC synaptosomes from CMS-V, indicating an increased localization of the receptor at synapses. MR synaptic enrichment in CMS-V appears to be cytoplasmatic, since CMS-V synaptic membranes showed no differences in MR levels.

Since MR acts as a sensor for shifts in CORT levels (*Joëls et al., 2012*), we can speculate that its increased localization at synaptic compartment could be involved in the response to the enhanced circulating levels of CORT seen in CMS-V.

Intriguingly, this effect was absent in CMS-V+KET, suggesting that KET treatment, although not reducing serum CORT levels, may be able to decrease MR responsiveness to CORT.

5.5.2 KET increases the transcriptional activity of GR

GR is a glucocorticoid receptor with less affinity for CORT than MR (*Gomez-Sanchez, 2014*). In the brain, GR is involved in the regulation of the stress response, by suppressing HPA axis activation and modulating MR activity (*Joëls et al., 2012*).

In line with a hypersecretion of CORT following chronic stressors, several studies have reported that CMS downregulates the expression of GR in HPC and PFC/FC (*Zheng et al., 2006; Xu et al., 2006*). However, this result does not seem to be consistent, since other studies failed to find the effect of CMS in GR expression in these brain areas (*Lopez et al.,*

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1998; van Riel et al., 2003; Hill et al., 2012). Accordingly, our results show that CMS had no effect on the total and synaptic expression of GR.

GR activity can be modulated by phosphorylation, an event which controls its activation, subcellular localization, transcriptional activity, and turnover (*Cattaneo and Riva, 2016*). Since it was previously shown that conventional antidepressants increase the phosphorylation of GR at Ser^{232} (*Anacker et al., 2011*), thus promoting its translocation to the nucleus and transcriptional activity (*Wang et al., 2002*), we also analyzed the levels of GR phosphorylated at Ser^{232}. While CMS exerted no effect on phospho-Ser^{232} GR levels, KET treatment was able to increase GR phosphorylation at Ser^{232}, both in HPC and PFC/FC. No effect of KET in phospho-Ser^{232} GR levels was found in synaptic fractions. Overall, these data suggest that KET may contribute to restore behavioral and functional changes in CMS-V by increasing GR-dependent transcriptional activity.

5.6 KET regulates the glutamate presynaptic machinery

5.6.1 Synapsin I phosphorylation is increased by CMS in CMS-R and by KET in CMS-V

Synapsin I is a presynaptic protein involved in vesicle mobilization (*Cesca et al., 2010*). In previous studies, we found that acute stress induces a rapid and sustained enhancement of depolarization-evoked release of glutamate in PFC/FC, by increasing the trafficking of glutamate presynaptic vesicles into the readily releasable pool, an event that requires the phosphorylation at Ser^9 of Synapsin I (*Treccani et al., 2014; Musazzi et al., 2016*).

In the present study, we investigated the possible role of phospho-Ser^9 Synapsin I in the changes induced by CMS and KET on glutamate presynaptic release.

Concerning the total levels of Synapsin I, we found no significant effect of CMS and KET. This result seems to be in contrast with previous studies in literature, showing that KET treatment increases the expression of Synapsin I (*Li et al, 2010; Müller et al., 2013*). However, it is worth noting that in these works, KET was administered only to naïve animals.

Our findings show instead that CMS and KET act on the phosphorylation of Synapsin I at Ser^9 in PFC/FC. Indeed, while in HPC we found no differences in phospho-Ser^9 Synapsin I

levels between experimental groups, increased phosphorylation of Synapsin I at Ser^9 was found in PFC/FC synaptosomes and synaptic membranes from CMS-R. Intriguingly, KET treatment exerted a similar effect in PFC/FC synaptic membranes from CMS-V.

However, regulation of the phosphorylation of Synapsin I at Ser^9, does not seem to be consistent with the changes induced by CMS and KET in presynaptic basal and evoked releases of glutamate.

5.6.2 KET rescues mGluR2 levels in CMS-V

mGluR2 is a presynaptic metabotropic glutamate receptor which negatively modulates glutamate release (*Chaki et al., 2013*). Recent evidence has associated mGluR2 with stress vulnerability. Indeed, it has been previously reported that mice exposed to chronic stress show decreased expression of mGluR2 in HPC and PFC/FC; however, while the decrease in PFC/FC is evident in all the animals subjected to stress, the reduction of mGluR2 in the HPC was selectively found in mice vulnerable to the stress paradigm (*Nasca et al., 2015*).

In agreement with these findings, we observed that CMS selectively induced a significant decrease of mGluR2 total and synaptic expression in the HPC of CMS-V rats. However, in contrast with previous results, we also found a reduction of mGluR2 expression in the PFC/FC of CMS-V.

The receptor has also recently gained attention as a promising target of novel antidepressants (*Chaki et al., 2013; Bruno et al., 2017*). In the present work, we found for the first time that acute treatment with KET was able to completely rescue the changes induced by CMS in mGluR2 levels, both in HPC and PFC/FC.

Although the functional outcome of these changes on glutamate neurotransmission remain elusive, our results further support the involvement of mGluR2 in stress susceptibility and antidepressant treatment.

5.7 CMS and KET exert no effect on mTOR activity

mTOR is a kinase involved in several critical cellular and neuronal functions (*Lipton and Sahin, 2014*). Among these, its induction of protein synthesis and synaptic plasticity have been recently associated to the rapid antidepressant action of KET (*Li et al., 2010; Li et al,*

2011). To investigate the role of mTOR expression and activation in the antidepressant effect of KET, we analyzed the total and synaptic protein levels of mTOR and phospho-Ser^{2448} mTOR.

Both CMS and KET exerted no significant effect on mTOR levels and phosphorylation. Although this result may seem in contrast with the previously reported activation of mTOR pathway induced by KET (*Li et al., 2010*), it should be noted that KET only transiently activates mTOR signaling, while the phosphorylation of mTOR was shown to disappear as fast as 1 hour after KET treatment.

5.8 KET rescues the activity of CaMKII in CMS-V

CaMKII is a synaptic protein involved in key synaptic functions, such as neurotransmitter synthesis and release, calcium homeostasis or synaptic plasticity, as well as in the regulation of gene transcription (*Lisman et al., 2002; Wang, 2008*).

While a previous work in literature showed that KET decreases the total levels of CaMKII (*Müller et al., 2013*), we found no significant effect of CMS and KET on the total expression of CaMKII. To possibly explain this discrepancy on CaMKII levels following KET treatment, it should be noted that in the work of Müller and collegues, KET was administered only to naïve animals.

Interestingly, we also found that CMS-V showed a significant reduction of CaMKII phosphorylation at Thr^{286} in HPC and PFC/FC, indicating a reduced activation of the enzyme. This change appeared only in total homogenate, and not in synaptic fractions, suggesting that CMS may mainly perturb the transcriptional activity mediated by CaMKII, rather than its synaptic function. Intriguingly, KET treatment was able to completely rescue the phosphorylation of CaMKII levels, possibly restoring its transcriptional activity. Since glutamate neurotransmission has a central role in the regulation of CaMKII function (*Lisman et al., 2002*), the reduced activation of CaMKII seen in the HPC of CMS-V is in line with the reduction of the basal and depolarization evoked glutamate releases observed in the HPC of CMS-V. Similarly, the rescue of CaMKII levels by KET parallels the rescue of basal glutamate release seen in HPC of CMS-V.

More difficult to explain is the divergence between CaMKII levels and glutamate presynaptic release in PFC/FC. Future studies will be needed to better understand a possible link between these two mechanisms.

5.9 CMS impairs the transcription of BDNF and BDNF splice variants

Several studies highlighted the central role of BDNF in both stress-related disorders and antidepressant treatments (*Castrén, 2004*). BDNF brain and plasma levels were found reduced in depressed patients (*Karege et al., 2005; Sen et al., 2008*), whereas a concomitant rescue of BDNF levels and structural changes was reported in cortical and limbic areas of patients following antidepressant treatments (*Chen et al., 2001; Castrén and Rantamäki, 2010*). Preclinical literature has shown that stress paradigms in rodents reduce BDNF-mediated signaling and structural complexity in the same brain regions affected in patients (*Duman and Monteggia, 2006; McEwen et al., 2016*). More recently, rapid translation of BDNF has been proposed to underlie the fast antidepressant effect of KET (*Kavalali and Monteggia, 2012; Kavalali and Monteggia, 2015*)

In the present work, we investigated the effect of CMS and KET on BDNF mRNA levels in HPC and PFC/FC. A high number of studies already assessed the effect of CMS on BDNF expression levels but, up to now, results do not seem to be consistent (*Hill et al., 2012*). Both reduced BDNF levels (*Nibuya et al., 1999; Hu et al., 2010*) or no changes (*Allaman et al, 2008*) have been found in the whole HPC. Similarly, in PFC/FC of rodents subjected to CMS, both reduced BDNF levels (*Xu et al., 2006*) or no changes (*Zhang et al., 2010*) have been reported.

As transcription of BDNF is regulated by at least 9 different promoters, each giving rise to a different splice variant transcript (*Sakata et al., 2009*), we also analyzed the effect of CMS and KET on the transcription of the BDNF splice variants mostly expressed in the rat brain: BDNF-1, BNDF-2, BDNF-4 and BDNF-6 transcripts.

Our results show that CMS significantly decreases the total mRNA levels of BDNF and BDNF splice variants in HPC of all stressed animals, including those treated with KET. We observed a similar effect also in PFC/FC, where total BDNF mRNA levels were found significantly decreased in all stressed animals in comparison to CNT. However, this brain

region showed less marked reductions in the mRNA levels of BDNF splice variants. Although the reason for this different effect of CMS in PFC/FC mRNA levels of BDNF splice variants is not clear, we can speculate that reductions in other BDNF variants should be taken into account for the decrease of total BDNF mRNA seen in PFC/FC.

It was previously proposed that resilience to CMS may be mediated by the expression of BDNF in HPC (*Taliaz et al., 2011*). Although this observation may seem in contrast with our results, we cannot exclude that CMS-R may preserve the expression and signaling of BDNF through other mechanisms (see section 5.10).

Moreover, it has been shown that KET antidepressant effect relies on BDNF translation. Although the evidence that KET exerted no effect on BDNF mRNA may seem surprising, it has already been reported that KET can rapidly stimulate the translation of BDNF independently of mRNA transcription (*Autry et al., 2011*). Thus, in line with previous findings, our data suggest that KET exerts its fast antidepressant effect without affecting BDNF transcription, at least in the 24 hours following injection.

5.10 CMS and KET modulate BDNF mRNA dendritic trafficking

We previously found that chronic treatment with classical antidepressants is able to increase the trafficking of BDNF mRNA to dendrites (*Baj et al., 2012*). Indeed, alternatively spliced BNDF transcripts can be targeted to distinct cellular compartments in neurons, ensuring BDNF mRNA availability, local translation, and synaptic function at distinct dendritic locations (*Tongiorgi, 2008; Baj et al., 2011*). This mechanism was suggested to support local translation of BDNF (*Tongiorgi, 2008*), and consequently to promote dendritic remodeling (*Verpelli et al., 2010; Baj et al., 2011; Kellner et al., 2014; Sun et al., 2014*).

In the present work, we analyzed for the first time the effect of CMS and KET on dendritic trafficking of total BDNF, BDNF-2 and BDNF-6 transcripts in HPC (discriminating between CA1 and CA3 subregions) and PFC/FC. Our results showed that dendritic trafficking of BDNF transcripts was particularly impaired in CMS-V, and at least partly rescued by acute KET.

Concerning CA1, we found that total BDNF mRNA dendritic trafficking was reduced in all CMS rats, although the reduction was more evident in CMS-V than in CMS-R, and was partly modulated by KET. The most striking results were found in CA3, where dendritic trafficking of total BDNF mRNA was selectively reduced in CMS-V, and not in CMS-R, and completely restored to control levels by acute KET. Similar reductions in CMS-V were also found for dendritic trafficking of BDNF-2 and BDNF-6 transcripts. CMS-R showed an increase of BDNF-2 transcript trafficking in CA1 and CA3, and of BDNF-6 transcript trafficking in CA1 and PFC/FC. Similarly, increased BDNF-2 transcript trafficking in CA1 and increased BDNF-6 transcript trafficking in PFC/FC was found in CMS-V treated with KET.

Overall, these findings suggest that the regulation of dendritic trafficking of BDNF may represent a critical mechanism underlying stress resilience and KET antidepressant effect. Indeed, the increased translocation of BDNF transcripts to dendrites reported in CMS-R (at least for BDNF-2 and BDNF-6 mRNAs) suggest that this may represent a compensatory mechanism for the reduction of total BDNF and BDNF splice variants mRNA levels seen in these animals.

Moreover, although acute treatment with KET showed no effect on mRNA levels of BDNF and BDNF splice variants, KET was anyway able to increase their trafficking to dendrites. Since it has been reported that KET can rapidly stimulate the translation of BDNF, without affecting mRNA transcription (*Autry et al., 2011*), we can hypothesize that the enhancement of BDNF dendritic trafficking induced by KET may result in increased synaptic availability of BDNF mRNA, thus supporting its local fast translation.

5.11 KET rescues dendritic atrophy of pyramidal neurons in CA3 of CMS-V

Due to the central role of BDNF in the regulation of structural plasticity and synaptogenesis (*Castrén, 2004*), we then studied whether the altered trafficking of BDNF mRNA observed in CA3 could correlate with changes of dendritic morphology in the same area. Previous studies using stress-based animal models of depression consistently showed that stress reduces dendritic complexity in cortical and limbic brain regions (*Duman and*

Monteggia, 2006; McEwen et al., 2016), including the CA3 area of HPC (*Magariños and McEwen, 1995*).

In line with previous evidence in literature, we found that CMS induced major morphological changes selectively in CA3 pyramidal neurons of CMS-V. Indeed, CMS-V showed reduced total length and number of branches of apical, but not basal, dendrites, and dendritic retraction of apical dendrites, effects which were absent in CMS-R.

This divergent effect of stress on apical and basal dendrites is consistent with previous studies in literature, showing that chronic stressors (*Magariños and McEwen, 1995*), as well as acute stress (*Nava et al., 2015*), induce dendritic retraction of apical dendrites in pyramidal neurons. Although the reason for this dissimilarity has not yet been clarified, it has been hypothesized that the different connectivity of apical and basal dendrites may underlie their distinctive sensitivity to stress (*Spruston, 2008; Conrad et al., 2017*).

While previous studies in literature already reported the ability of KET to reverse dendritic remodeling and loss of synapses induced by stress (*Li et al., 2011; Duman and Aghajanian, 2012*), no studies up to date have investigated the effect of KET on HPC dendritic morphology following stress exposure.

In the present work, we showed for the first time that a single injection of KET, in just 24 hours, is able to completely reverse the changes induced by CMS in dendritic complexity of CA3 pyramidal neurons.

Although our data do not provide a clear connection between dendritic morphology and BDNF mRNA dendritic trafficking, it is worth noting that the morphological changes observed in CA3, are in line with the changes in BDNF mRNA dendritic trafficking.

5.12 Conclusions

In the present work, we showed that most of the behavioral, functional and morphological changes induced by CMS in the HPC of vulnerable animals were completely rescued by an acute treatment with KET.

CMS induced anhedonia in nearly half of the rats subjected to the stress paradigm. According with their depressive-like behavior, CMS-V exhibited marked phenotypic changes, including reduced body weight gain and increased weight of adrenal glands and CORT serum levels, in line with a dysregulation of the HPA axis.

The hedonic behavior of CMS-V rats was completely rescued by a single sub-anesthetic dose of KET in just 24 hours.

Using the technique of the isolated synaptic terminals in superfusion, we were able to find that CMS reduced basal and depolarization evoked releases of glutamate in the HPC of CMS-V. KET treatment restored basal, but not depolarization-evoked, glutamate release in CMS-V, suggesting a rescue of synaptic homeostasis.

We also found that CMS induced several modifications in the expression and phosphorylation levels of different proteins involved in glutamate release and stress response, including increased synaptic localization of MR, decreased expression of mGluR2 and decreased activation of CaMKII. All these changes were reversed by acute KET.

CMS-V showed decreased levels and dendritic trafficking in total-BDNF and BDNF splice variants transcripts in HPC. KET treatment, although not reversing changes in BDNF mRNA levels, completely rescued dendritic trafficking in CA3 of CMS-V. Accordingly, CA3 pyramidal neurons of CMS-V showed a reduction in total length and branching of apical dendrites. KET completely restored morphological changes.

In conclusion, our results confirm the fast antidepressant action of KET, both at molecular, neuronal and behavioural levels.

Hopefully, further investigation of the mechanisms underlying individual resilience or vulnerability to stress and fast KET antidepressant action will help to clarify the neurobiological underpinnings of depression and to identify new pharmacological targets for faster, more efficient antidepressant drugs.

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