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THE GLUTAMATE HYPOTHESIS OF DEPRESSION: THE EFFECT OF STRESS AND GLUCOCORTICOIDS ON GLUTAMATE SYNAPSE AND THE ACTION OF ANTIDEPRESSANTS

Doctorate thesis

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ABSTRACT

In the last years, a consistent number of clinical and preclinical studies have demonstrated that glutamatergic transmission has a primary role in the pathophysiology of mood and anxiety disorders (MADI). It has been shown that in depressed patients the levels of glutamate and its metabolites are altered in plasma and in selected brain areas and mRNA and protein levels of glutamate receptors are changed in brain areas. A number of preclinical studies on animal models of MADI have shown that different types of environmental stress and glucocorticoid administration affect glutamate transmission and exert structural brain remodeling in the same areas involved in human pathology. These effects of stress and glucocorticoids have been associated with the onset and exacerbation of neuropsychiatric disorders.

In previous studies we found that acute footshock (FS)-stress induces an increase of glutamate release from synaptosomes of prefrontal and frontal cortex (PFC/FC), via glucocorticoid receptor (GR) activation and SNARE complexes accumulation in synaptic membranes. Furthermore, we have demonstrated that the increase of glutamate release induced by acute stress is prevented by chronic antidepressants (ADs). Additional studies have also shown that ADs can regulate glutamate transmission through glutamate receptors; reducing the function of NMDA receptors, potentiating the function of AMPA receptors and affecting different subtypes of metabotropic glutamate receptors. Together, these findings have identified the glutamate synapse as a target for novel glutamatergic ADs.

Considering the importance of stress-induced alteration of presynaptic glutamate release in the pathophysiology of MADI, we

aimed to study whether the enhancement of depolarization-evoked glutamate release induced by acute stress was related to an increase of the readily releasable pool (RRP) of vesicles and whether this effect was mediated by a synaptic non-genomic action of corticosterone (CORT).

We found that FS-stress increased glutamate release evoked by hypertonic sucrose (which mobilizes exclusively the RRP), suggesting an increase in the RRP size. Then we found that this synaptic effect of stress was dependent on local CORT action. Indeed, CORT was able to directly affect the RRP size through the activation of GR and mineralcorticoid receptors (MR). The preincubation with RU486, a selective GR antagonist, and spironolactone, a selective MR antagonist, prevented the CORT-induced increase of RRP. Contrary to acute stress, CORT by itself did not promote vesicle fusion, since CORT application *in vitro* did not increase glutamate release evoked by depolarization in control synaptosomes, and did not affect excitatory post-synaptic potentials and paired pulse facilitation in medial PFC slices. Furthermore, we found that CORT increased vesicle mobilization towards the RRP via GR and MR activation, by using total internal fluorescence microscopy, a technique that allows the study of events occurring in a 100 nm-interval below the plasma membrane.

Finally we found that stress and CORT modulated synapsin I, a protein involved in vesicle mobilization and in vesicles docking, fusion and recycling at active zones. We found that both FS-stress and CORT induce an increase of synapsin I phosphorylation in synaptic membranes selectively at site 1. The preincubation with

both RU486 and spironolactone, prevented the CORT-induced synapsin I phosphorylation at site I, suggesting that this protein is involved in the pathway downstream of activation of the two receptors.

Together these results suggest that the increase of the RRP size induced by stress is promoted by a local action of CORT on synaptic receptors. We speculated that CORT is necessary to promote an increase of the RRP, but not sufficient to increase depolarization-dependent glutamate release, suggesting that additional mediators or neurotransmitters released during the stress response, are necessary to trigger vesicle release.

INTRODUCTION

1- Depression

Depression is a complex neuropsychiatric pathology and the fourth leading cause of disability and disease worldwide. The World Health Organization projections indicate that depression will be the highest ranked cause of disease in the middle- and high-income countries by the year 2030 (Mathers and Loncar, 2006; Musazzi et al., 2012).
The diagnosis of depression is subjective and essentially based on meeting criteria spelled out in the Diagnostic and Statistical Manual of Mental disorders (DSM-IV-TR). The symptoms that must persist for a 2-week period include depressed mood, anhedonia (reduced ability to experience pleasure from natural rewards), irritability, difficulties in concentrating, and abnormalities in appetite and sleep (neurovegetative symptoms) (Nesler et al., 2002, Krishnan and Nestler, 2008).
Despite the huge number of clinical and preclinical studies and considering the prevalence of depression and its social impact, there is a lack of knowledge regarding the pathophysiology of the disease. The first explanation is that many brain regions are involved in the pathology and responsible for the different symptoms of depression. Indeed, neocortex and hippocampus may mediate cognitive aspects of depression, such as memory impairments and feelings of worthlessness, hopelessness, guilt, doom and suicide. Striatum (ventral striatum and nucleus accumbens) and amygdala are involved in emotional memory and could mediate anhedonia and anxiety observed in patients. For neurovegetative symptoms, the involvement of the hypothalamus has been proposed (Nestler et al., 2002). The second reason comes from the complex etiology of the

pathology. It has been proposed that the depressive phenotype is the outcome of a combination of different genetic and environmental factors (Figure 1) (Wong and Licinio, 2011). Indeed, epidemiological and clinical studies support the hypothesis that a genetic predisposition associated with psychological perturbations, in particular during the neonatal period (see below), may result in a phenotype that is vulnerable to additional exposure to stress, leading to development of depression in adult life (Heim and Nemeroff, 2001; Heim et al., 2004).

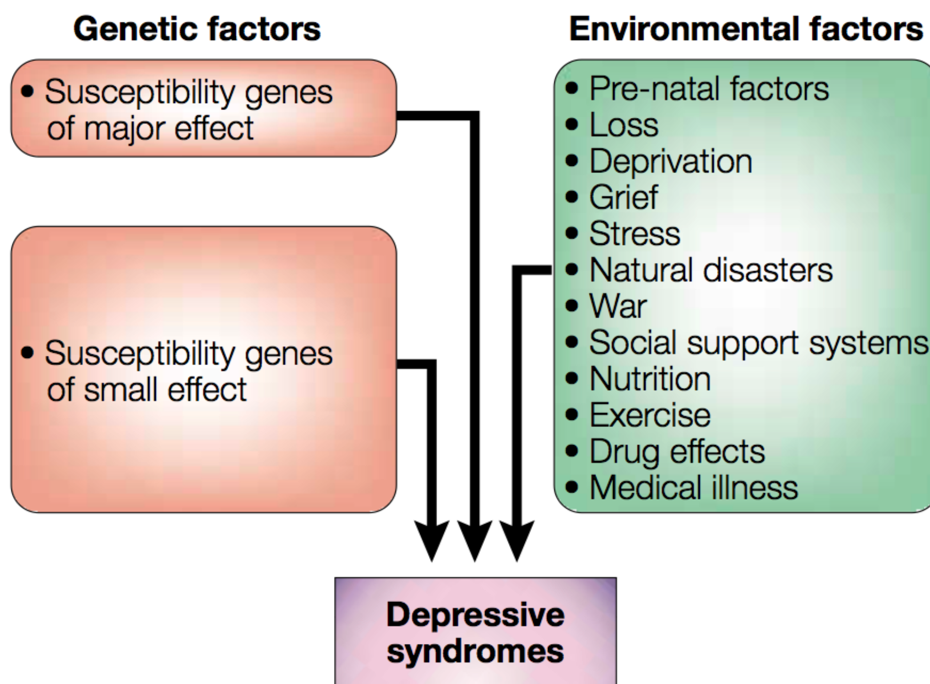

Figure 1. Interaction between genetic and environmental factors at the basis of depression *(from Wong and Licinio, 2001).*

A known example of gene-environment interaction in depression is represented by the polymorphism in the promoter region of the serotonin transporter (5-HTT) gene (Caspi et al., 2003). The 5-HTT that is involved in the reuptake of serotonin at brain synapses, is blocked by some second-generation antidepressants drugs (selective serotonin reuptake inhibitors and serotonin and noradrenaline reuptake inhibitors). The 5-HTT is encoded by a single gene, which has an allelic variation in the promoter region: the shorter allele “s” is responsible of a reduced transcriptional efficiency, compared with the longer one “l”. Individuals with one or two copies of the “s” allele of the 5-HTT promoter exhibit more depressive symptoms, diagnosable depression, and suicidality in relation to stressful life events more frequently than individuals homozygous for the “l” allele (Caspi et al., 2003). Although the genetic background plays a key role in depression, environmental factors seem to work as the main triggering events (Caspi and Moffitt, 2006). A number of early-life stress events were related with the onset of mental disorders, particularly maternal stress and substance abuse during pregnancy, low birth weight, birth complications, deprivation of normal parental care during infancy, childhood physical maltreatment, childhood neglect, premature parental loss, exposure to family conflict and violence, stressful life events involving loss or threat, substance abuse, toxic exposures and head injury (Caspi and Moffitt, 2006).

For these reasons the study of the mechanisms activated within the brain in response to environmental factors, in particular to stress and

to the stress hormones corticosteroids, has received high relevance to understand the etiopathogenesis of depression.

2- The stress response

All living organisms are in a dynamic metabolic equilibrium, which is called homeostasis. This equilibrium could be altered by physical and psychological events that are known as “stressors”, which are defined as events or experiences that interfere with the ability of an individual to adapt and cope (de Kloet E. R. et al., 2005, Popoli et al., 2012). As a consequence, the stressor evokes a physiological stress response, which involves the release of hormones and mediators that can promote adaptation, when the response is promptly turned off. However, if the response is dysregulated, it may promote pathological processes (Popoli et al., 2012; McEwen, 1998). Two systems are primarily involved in the stress response: the first one involves the rapid activation of the sympathetic nervous system, which leads to the release of adrenaline and noradrenaline from the adrenal medulla. The parasympathetic nervous system is consequently activated to prevent overshooting (Joëls et al., 2012). The second system involved in the stress response is the HPA axis.

2.1 The HPA axis

The main mechanism by which the brain reacts to stress is the activation of the HPA axis (Nestler et al., 2002) (Figure 2).

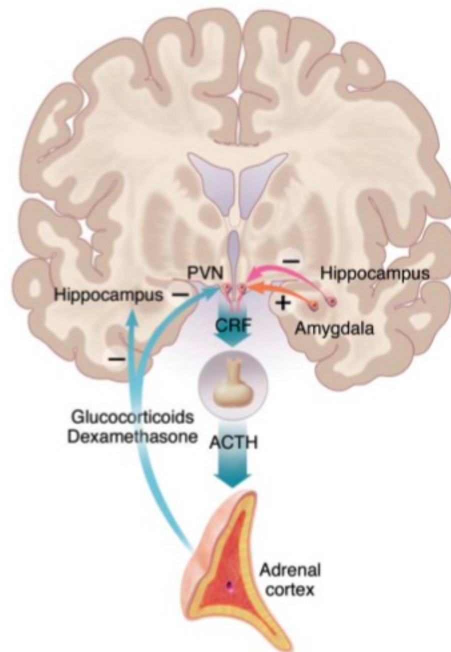

Figure 2. The HPA axis *Parvocellular neurons of the PVN produce CRH and vasopressin, which upon stress are released into the portal vessels. CRH and vasopressin reach the anterior pituitary gland, where their actions lead to secretion of ACTH into the circulation. In turn, this gives rise to the synthesis and release of glucocorticoids from the adrenal cortex, which exert a negative feedback on CRF and ACTH synthesis and release. The HPA axis is controlled by hippocampus (inhibitory effect) and amygdala (excitatory input) (from Nestler et al., 2002; see text for details).*

Parvocellular neurons in the middle part of the paraventricular nucleus (PVN) produce corticotrophin-releasing hormone (CRH) and vasopressin, which upon stress are released in high amount from terminals at the median eminence into the portal vessels. Through these vessels, CRH and vasopressin reach the anterior pituitary gland, where their actions lead to secretion of adrenocorticotropin

hormone (ACTH) into the circulation (Joëls et al., 2012). In turn, ACTH stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone, CORT, in rodents) from the adrenal cortex. Blood concentration of adrenal glucocorticoids rise to peak level after 15-30 minutes and then decline slowly to pre-stress levels 60-90 minutes later (de Kloet et al., 2005).

The activation of the HPA axis is controlled by different brain areas including hippocampus and amygdala. The hippocampus exerts an inhibitory influence on hypothalamic CRF-containing neurons via a polysynaptic circuit, while amygdala gives a direct excitatory input (Nestler et al., 2002; see Figure 2). On the other hand, glucocorticoids exert a powerful negative feedback effect on the HPA axis, by regulating hippocampal and PVN neurons. In mammals, these hormones are released from the adrenal glands in a daily circadian cycle. Indeed, in humans the circulating levels of cortisol are low in the morning and progressively increase, reaching a peak at the end of the resting phase. However, high resolution blood sampling methods have shown that these circadian fluctuations overlay a highly oscillatory ultradian pattern, with a periodicity of approximately 60 minutes (Joëls et al., 2012). In animal models, it has been recently proposed that this ultradian hormone secretion induces glucocorticoid receptor-mediated pulses of gene transcription (Stavreva et al., 2009).

2.2 Corticosteroid receptors

In the brain, glucocorticoids exert their function through the activation of two types of receptors: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (Reul and de Kloet, 1985). The name of the two receptors derive from to the main peripheral process in which they are involved: mineral balance and gluconeogenesis, respectively. Classic MR and GR belong to the superfamily of nuclear receptors, which act as transcriptional factors. The human GR gene is constituted by nine exons, among which exons 2 to 9 encode for the GR protein (Joëls et al., 2012). Exons 1 and 9, can be alternatively spliced generating different mRNAs. Alternative splicing of the non-coding exon 1 produces different variants responsible for the region- and tissue-specific expression patterns, while the splicing of exon 9 produces two isoforms, GRα and GRβ. Also the MR mRNA can be alternatively spliced, but less is known about the different isoforms of the receptor (Joëls et al., 2012). The expression of MR and GR varies in different brain regions and cells (Reul and de Kloet, 1985): GR are expressed both in neurons and in glial cells, and they are particularly expressed in PVN, in CA1 and dentate gyrus of hippocampus, in amygdala and in lateral septum, while MR are mainly expressed in neurons of hippocampus and lateral septum (Joëls et al., 2012). Moreover, GR and MR show a different affinity for endogenous hormones (corticosterone, cortisol and aldosterone) which in turn lead to a variation in MR and GR activity depending on hormone concentrations in the brain. MR have higher affinity for the endogenous hormones aldosterone, cortisol and CORT, with a K_d of

~ 0.5 nM (Reul and de Kloet, 1985), while GR have a 10-fold lower affinity for CORT, and cortisol and many-fold lower for aldosterone. Considering the high affinity of MR for cotisol/CORT and the physiological higher level of cortisol/CORT in respect to aldosterone, in the brain MR in basal conditions are substantially occupied by cortisol/CORT, even during the intervals between ultradian pulses and in the absence of stress (Reul and de Kloet, 1985). In contrast, GR are partly occupied when corticosteroid levels are low and gradually become occupied when hormone levels rise (e.g. after stress) (Joëls et al., 2012).
As reported in table 1, the two receptors have different affinity also for synthetic compounds (selective and non-selective agonists and antagonists) used both in clinic and research.

Table 1. Pharmacological profile of human GR and MR

RECEPTOR	AGONISTS (Affinity)	ANTAGONISTS
GR	Dexametasone>Triamcinolone> Prednisolone> Cortisol> Corticosterone= Deoxycorticosterone	Mifepristone
MR	Deoxycorticosterone= Progesterone> Fludrocortisone> Aldosterone>Cortisol>Dexametasone	Drospirenone Eplerenone Spironolactone

2.2.1 Classical cytosolic corticosteroid receptors

The most common mechanism of action of GR and MR implies the activation of genomic pathways. Circulating glucocorticoids diffuse through cell membranes and bind intracellular MR and GR. The receptors bind to a multiprotein complex of heat shock proteins (Hsp90, Hsp70, Hsp56, and Hsp40) and form activated receptor complexes. This induces a conformational modification allowing homodimerization or heterodimerization of activated receptors with the consequent dissociation from heat shock proteins. Dimerized MR and GR translocate into the nucleus, where they bind to consensus sequences in the promoter of responsive genes, directly modulating their transcription. On the other hand, GR or MR monomers can interact with other transcription factors (nuclear factor-kappa B (NFkB), activator protein 1 (AP1) or cyclic AMP response element-binding (CREB)), inhibiting their activity leading to transrepression (Datson et al., 2008). Genomic effects are slow in onset, and the initial physiological responses start at least with a 15 min-delay, and sometimes even hours after the beginning of stress (Groeneweg et al., 2012). However, a growing number of recent evidence demonstrated that, in neurons, corticosteroids, through GR and MR activation, have also fast effects, which have been observed within seconds to minutes, and which can not be explained through the activation of genomic pathways (Karst et al., 2005; reviewed in Groeneweg et al., 2012).

2.2.2 Novel membrane-associated corticosteroid receptors

To explain the rapid effect of corticosteroids, recent studies have shown that CORT also produces rapid non-genomic effects on neuronal excitability through the activation of the same GR and MR described above, but presumably associated to the plasma membrane. The presence of MR and GR has been shown by Western blot analysis in synaptosome extracts (Komatsuzaki et al., 2005; Wang and Wang, 2009) and at neuronal membranes using electron microscopy (Johnson et a., 2005; Prager et al., 2010). In particular, it seems that, in amygdala, MR are more expressed pre-synaptically, whereas GR are more expressed post-synaptically (Prager et al., 2010). The involvement of classical MR and GR in mediating rapid nongenomic effect on neuronal excitability was proved in different brain areas (see below) and using both antagonists and knockout mice for the MR and GR (Karst et al., 2005, 2010, see also above). However, the molecular mechanism by which the receptors interact or translocate to the plasma membrane is still unknown.

For another steroid receptor (the estrogen receptor α), it was demonstrated that it can be inserted into membrane through palmitoylation (Pedram et al., 2007). Starting from this evidence, it has been shown that GR have a conserved palmitoylation motif (Pedram et al., 2007), which could be responsible for the trafficking and binding of the receptor toward neuronal membranes.

However, other authors, starting from the experimental evidence that G-protein inhibitors can prevent many corticosteroid effects

independently from MR and GR activation, support the hypothesis that CORT can also bind to a G-protein coupled receptor (Di et al., 2009; Groeneweg et al., 2012, Olijslagers et al., 2008). Nevertheless, literature data are conflicting and this novel receptor has not been yet identified and cloned yet (Groeneweg et al., 2012).

2.3 Rapid corticosteroid effects in the brain

Different studies have been performed to understand the fast effects of CORT on neuronal excitability and in cognitive processes. The three brain regions in which these effects have been more characterized are hippocampus, amygdala and hypothalamus.

The high majority of studies in hippocampus focus on the rapid effect of CORT on neuronal excitability at pre- and postsynaptic level. It has been shown that CORT, after binding to presynaptic MR and activating the ERK1/2-MAP kinase signaling, promotes a rapid increase in the frequency of the miniature excitatory postsynaptic currents (mEPSCs), indicating an increase in the release probability of glutamate (Karst et al., 2005; Olijslagers et al., 2008). Moreover, it was shown that the hormone activates also postsynaptically located MR, which seem to be G-protein-coupled, which activate ERK1/2 that, in turn, via phosphorylation of potassium channels, inhibit the repolarizing postsynaptic I_A-currents. Since the current work to dampen excitatory inputs, the CORT-induced decrease of I_A-currents could lead to an increase in the probability of the generation of the postsynaptic action potential (Olijslagers et al., 2008). It has been shown that, at postsynaptic level, CORT also changes glutamate

transmission increasing lateral movement of GluA2 subunits of the AMPA receptors, via MR activation, therefore modulating the plastic range of glutamatergic synapses through a change in the AMPA receptor surface trafficking (Groc et al., 2008).

As for hippocampus, neurons in the basolateral amygdala (BLA) show an increase in mEPSCs frequency dependent on MR after acute CORT exposure (Karst et al., 2010). Moreover, selectively in BLA, the effects of CORT are long lasting and greatly affect the responsiveness to CORT, since a subsequent pulse of CORT induces a GR-dependent decrease of the mEPSCs frequency. Similarly, it was shown that the acute *in vitro* stimulation with CORT of BLA slices from acute stressed animals (20 minutes restraint stress) activates an endocannabinoid signaling pathway that suppresses mEPSC frequency via GR-dependent pathway (Karst et al., 2010).

On the contrary, in parvocellular neurons of the PVN, the acute exposure to CORT decreases the release probability of glutamate, via activation of GR and involvement of retrograde endocannabinoid signaling. At the same time, CORT enhances GABAergic miniature inhibitory postsynaptic currents (mIPSCs) through retrograde nitric oxide signaling (Di et al., 2003; Di et al., 2009).

Compelling evidence suggests that the fast effect of glucocorticoids also plays a role in cognitive processing. Interestingly, it has been shown that a GR agonist affects memory functions mainly acting on the medial PFC (mPFC), via the activation of a membrane-bound steroid receptor, increasing levels of cAMP-dependent protein kinase

(PKA) through modulation of noradrenergic activity (Barsegyan et al., 2010).

2.4 Delayed effects of corticosteroids

Although there is an increasing knowledge regarding the non-genomic effects of CORT, the high majority of studies reported in literature concerns the gene-mediated effect of CORT. These effects have been particularly investigated in hippocampus, BLA and mPFC. As an example, in hippocampal slices, a pulse of CORT enhances the amplitude but not the frequency of mEPSCs recorded 1-4 hours after corticosteroid exposure, via cytosolic GR activation (Karst and Joëls, 2005; Martin et al., 2009). The effects on mEPSCs amplitude were shown to peak between 150 and 200 minutes and were not seen earlier than 1 h after CORT delivery (Karst and Joëls, 2005; Martin et al., 2009). A similar enhancement in mEPSC amplitude has been also reported for prelimbic neurons in the PFC after acute stress exposure. Indeed, it has been shown that, more than 1 hour after cessation of stress there is an increase in NMDA and AMPA receptors-mediated synaptic currents, sustained for 24 hours and mimicked by short-term CORT treatment *in vitro.* The delayed enhancement of glutamate transmission induced by acute stress and CORT was related with an increased surface expression of NMDARs and AMPARs at the postsynaptic plasma membranes, consequent to intracellular GR activation, and induction of serum- and glucocorticoid-inducible kinase (SGK) and Rab4 (Yuen et al., 2009; Yuen et al., 2011).

2.5 Chronic corticosteroid exposure

As reported above, acute corticosteroid exposure (minutes to hours) modulates glutamate transmission and enhances synaptic function. On the other hand, chronic exposure to corticosteroids (days to weeks) mediates adaptative plasticity, involving spine synapse turnover and dendritic shrinkage (Popoli et al., 2012; McEwen, 1999). These effects are strongly depending from the considered brain regions: many studies have been performed in hippocampus, amygdala, and to a minor extent, in PFC. For example, it has been shown that chronic unpredictable stress (CUS), an animal model of depression, causes a reduction in the length and branching of apical dendrites and decreases the number and function of spine synapses of pyramidal neurons in layer V of the mPFC (Li et al., 2011). Other chronic stress paradigms (such as restraint stress) cause similar reductions in dendrite complexity and spine density in PFC neurons and CA3 pyramidal cells of the hippocampus (Duman and Aghajanian, 2012; Joëls et al., 2012). Chronic stress also suppresses adult neurogenesis in the adult hippocampus and significantly reduces the number of glial cells in the mPFC (Duman and Aghajanian, 2012). On the other hand, increased dendritic complexity has been reported in principal cells of BLA and in the orbital cortex (Joëls et al., 2012, Roozendaal et al., 2009) (Figure 3), suggesting a different role of chronic corticosteroid exposure in these areas.

These morphological alterations seem to be reversible with the cessation of stress (Conrad et al., 1999; Radley et al., 2005) except

for the BLA, where changes persisted for at least 30 days after chronic stress (Vyas et al., 2004). These processes were also linked to the age of animals, indeed the mPFC of aged animals show a slower recovery than for younger animals (Bloss et al., 2010).

Structural plasticity process can also be activated after acute stress in amygdala. A single traumatic stressor causes BLA neurons to grow new spines over the next 10 days, with no growth of dendrites (Mitra et al., 2005). Moreover, a single, high dose of injected CORT causes delayed dendritic growth over the next 10 days (Mitra and Sapolsky, 2008), even if no data are available on possible effects on the number of spines.

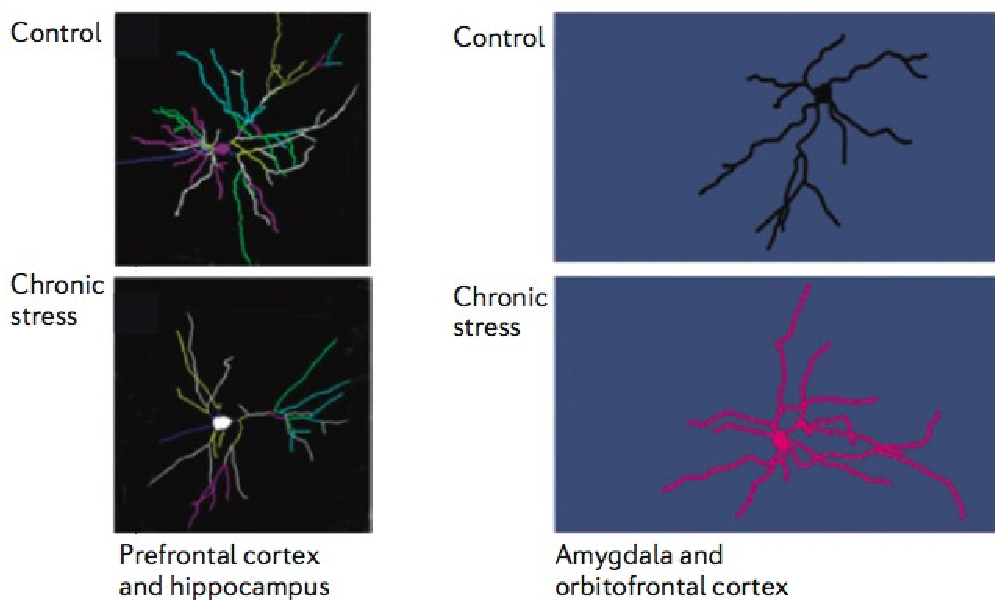

Figure 3. Structural and morphological changes induces by stress in prefrontal cortex, hippocampus and amygdala *(from Popoli et al., 2012).*

These morphological changes are expected to affect neuronal activity. In particular, in hippocampus, it was shown that chronic overexposure to stress hormones causes a reduction in the ability to induce or maintain long-term potentiation (LTP) and enhances the probability to induce long-term depression (LTD) (Joëls et al., 2012). Two different models have been proposed to explain the complex effects of chronic stress on neuronal morphology and excitability in hippocampus. The first one proposes that, at least in the CA3 hippocampal area, the increase of glutamatergic transmission consequent to chronic stress exposure leads to excitotoxic effects inducing dendritic atrophy and reduction in spine number (McEwen, 1999). This mechanism could be interpreted as a “protective” mechanism by which cells, through reduction in the number of synaptic contacts, tries to counteract the enhanced excitatory input. In support of this theory, treatment of animals with NMDA receptor blockers was found to prevent dendritic remodeling in the CA3 area of HC and in mPFC (McEwen and Magarinos, 1997; Christian et al., 2011; Li et al., 2011). According to this theory, enhanced excitatory transmission would precede dendritic retraction rather than occur simultaneously or as a consequence (Joëls et al., 2012).

Conversely, the second model, mainly based on experimental and mathematical evidence, suggests that dendrite remodeling and altered synaptic excitability, observed in the hippocampus after chronic stress, lead to atrophy-induced synaptic hyper excitability that could tilt the balance of plasticity mechanisms in favor of synaptic potentiation over depression. Indeed, it has been shown that chronic stress enhances NMDA receptor-mediated EPSCs in HC

CA3 neurons (Kole et al. 2002; 2004) and that DG granule cells from chronic stressed animals, when exposed to CORT in vitro, show an increase in the amplitude of AMPA receptor-mediated synaptic currents (Karst and Joëls, 2003). These larger currents were not found in cells after chronic stress only or acute CORT treatment. Similarly, it has been demonstrated using biophysical models of hippocampal CA3 neurons that dendritic atrophy leads to an amplification of intrinsic and synaptic excitability, suggesting that stress may impair learning and memory through a facilitation of intrinsic synaptic excitability and the consequent imbalance of bidirectional hippocampal synaptic plasticity (Narayanan and Chattarji, 2010).

The effects of chronic stress and corticosteroid exposure have been recently studied also in PFC. In this area, it has been shown that while acute stress enhances glutamate transmission and related cognitive function, chronic stress impairs these processes. Indeed, 5 to 7 days of restraint or unpredictable stress in young rats causes a reduction of both AMPA and NMDA receptor-dependent synaptic responses in pyramidal PFC cells, in association with ubiquitin/proteasome mediated degradation of selective subunits (Yuen et al., 2012).

3 Stress as a risk factor for neuropsychiatric disorders

The different effects of CORT depending on the age of the animal, the time of exposure and the duration and type of stressor experienced, underline how difficult is to clearly understand the

mechanisms by which CORT is able to modulate neuronal excitability and plasticity. A number of preclinical studies suggest that stress can produce in animals some of the cognitive and emotional disturbances that are also observed in patients with depression. Several animal models involving different forms of stress have been used for studying the etiopathogenesis of depression. One of the more recent models is chronic unpredictable stress CUS, where animals are exposed to a variable sequence of mild, unpredictable stressors (Willner, 2005). CUS was shown to increase blood levels of CORT and behavioral abnormalities, including core symptoms of depression, such as anhedonia (Li et al., 2011).

The importance of the functionality of the HPA-axis comes also from animal models in which components of the HPA axis were modified by mutagenesis (de Kloet et al., 2005). For example, GR-knockout mice generated by deletion of limbic GR (except for the hypothalamic PVN), exhibit a robust depression-like phenotype (Boyle et al., 2005). It was also clearly demonstrated that the correct functionality of the HPA-axis plays an important role in the etiology of depression also in humans. First, it has been shown that several individuals with depression exhibit hyperreactivity of the HPA-axis, even before the onset of any clinical symptom (Joëls et al., 2010). Second, several depressed patients show elevated circulating corticosteroid levels (especially during the circadian trough) and relative insensitivity to dexamethasone-induced suppression of the HPA-axis. The normalization of these functions generally precedes relief of depressive symptoms and the degree of normalization predicts the probability of relapse (Joëls et al., 2011). Third, it has been shown

that antiglucocorticoids (given in addition to antidepressants) to patients with psychotic depression accelerate and increase chances of successful treatment (Joëls et al., 2011). Since the functionality of corticosteroid hormones and receptors, as well as the HPA axis in general can be affected by mutations, the genetic background may also predict the probability that individual patients will respond to pharmacotherapy. For instance, it was demonstrated that a specific mutation in the FKBP5 gene confers a faster response to antidepressants compared with the wild type (Binder et al., 2004). This gene encodes for a co-chaperone of HSP90 and contributes to the folding of cytosolic GR, determining the affinity of cortisol for its receptor.

Other genetic factors might also cause individuals to be resilient in the developing of affective disorders. An example is the polymorphism in the ER22/23 EK allele, which is located at the beginning of exon 2 of the GR gene and confers a healthier metabolic profile and a better cognitive function than the general population. The polymorphism is also associated with a better treatment outcome in individuals with depression (de Kloet et al., 2005; van Rossum and Lamberts, 2004). It remains a challenge for the future to study the consequences of these genetic variants on corticosteroid-dependent modulation of neuronal activity (Joëls et al., 2012).

4 New approaches in neuropsychopharmacology: the glutamatergic hypothesis of depression

As previously shown corticosteroid and stress exert crucial effects on neuronal excitability and brain functions through rapid and delayed mechanism. Moreover, it has been demonstrated that the modulation of excitatory, glutamatergic transmission by CORT plays a critical role in the stress response and has a specific effect depending on the brain region involved. These effects on glutamate transmission are predominant in hippocampus, amygdala and PFC, all regions involved in depression. Abnormal function of glutamatergic transmission has been reported also in neuropsychiatric diseases, including depression. Indeed, it has been shown that the levels of glutamate and its metabolites are altered in plasma and in selected brain areas of patients affected by mood and anxiety disorders (Hashimoto et al., 2007; Küçükibrahimoglu et al., 2009, Yüksel and Öngür, 2010). Moreover, post-mortem studies have found alterations of mRNA and protein levels of glutamate receptors in brain areas from depressed individuals (Beneyto et al., 2007). Several studies have also investigated the role of glial cell that participate in the uptake, metabolism and recycling of glutamate and that have been proposed to be involved in the alterations of glutamate neurotransmission observed in depression. It has been shown that the expression of the glial excitatory amino acid transporters was reduced in individuals with mood disorders (Choudary et al., 2005), and that glial cell number and/or density is reduced in the brain

regions with glutamatergic predominance from patients with major depression (Rajkowska et al., 1999).

All these findings suggest that mood disorders are associated with abnormal function and regulation of the glutamatergic neurotransmitter system (Sanacora et al., 2008). For this reason the study of the mechanisms by which glutamate transmission can be modulated, particularly by stress and glucocorticoids, could play an important role in the development of new fast-acting antidepressants. Considering the glutamatergic synapse as a tripartite structure (Figure 4), corticosteroid and stress can affect different mechanisms including glutamate release, glutamate receptors and glutamate clearance and metabolism (Popoli et al., 2012). The identification of the mechanisms by which corticosteroids regulate the functions of the glutamate synapse and the mechanisms by which antidepressants can modulate glutamate transmission will provide the opportunity to use novel pharmacological interventions to improve and preserve neural function and to treat and possibly prevent some psychiatric disorders (Popoli et al., 2012).

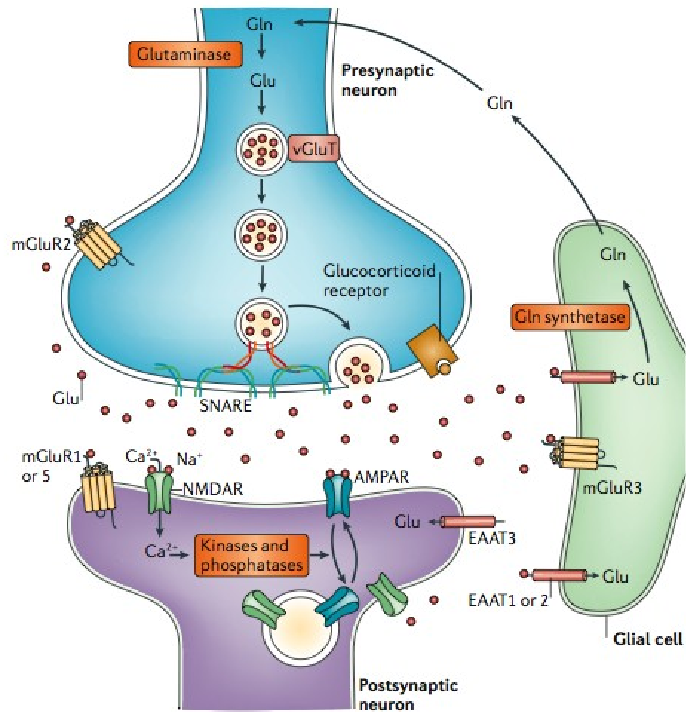

Figure 4. The glutamate tripartite synapse *The figure shows the organization of glutamate synapse. Within the synapse glutamatergic transmissions is controlled at different points: glutamate release, postsynaptic receptor trafficking and function, transporter-mediated uptake and recycling of glutamate through the glutamate-glutamine cycle (from Popoli et al., 2012)*

CHAPTER I

Biological Psychiatry (in press)

The action of antidepressants on the glutamate system: regulation of glutamate release and glutamate receptors

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Abstract

Recent compelling evidence has suggested that the glutamate system is a primary mediator of psychiatric pathology and also a target for rapid acting antidepressants. Clinical research in mood and anxiety disorders has shown alterations in levels, clearance and metabolism of glutamate, and consistent volumetric changes in brain areas where glutamate neurons predominate. In parallel, preclinical studies with rodent stress and depression models have found dendritic remodeling and synaptic spines reduction in corresponding areas, suggesting these as major factors in psychopathology. Enhancement of glutamate release/transmission, in turn induced by stress/glucocorticoids, seems crucial for structural/functional changes. Understanding mechanisms of maladaptive plasticity may allow to identify new targets for drugs and therapies.

Interestingly, traditional monoaminergic-based antidepressants have been repeatedly shown to interfere with glutamate system function, starting with modulation of NMDA receptors. Subsequently, it has been shown that antidepressants reduce glutamate release and synaptic transmission, in particular it was found antidepressants prevent the acute stress-induced enhancement of glutamate release. Additional studies have shown that antidepressants may partly

reverse the maladaptive changes in synapses/circuitry in stress and depression models. Finally, a number of studies over the years have shown that these drugs regulate glutamate receptors, reducing the function of NMDA receptors, potentiating the function of AMPA receptors, and, more recently, exerting variable effects on different subtypes of metabotropic glutamate receptors. The development of NMDA receptor antagonists has opened new avenues for glutamatergic, rapid acting, antidepressants, while additional targets in the glutamate synapse await development of new compounds for better, faster antidepressant action.

Introduction

Glutamate neurotransmission dysfunction is increasingly considered a core feature of stress-related mental illnesses. For over half a century the conceptual framework of research on these disorders has been dominated by the monoamine hypothesis, on which most of the drugs developed for clinical therapy are based. Although it was not acknowledged as a neurotransmitter until the early 1980s (1), glutamate has been later recognized as the major excitatory neurotransmitter in the brain, with glutamatergic neurons representing about 80% of total neurons in neocortex (2,3). In the

past decade it has become increasingly acknowledged that maladaptive changes in the structure and function of excitatory/inhibitory circuitry (representing the vast majority of neurons and synapses in brain) have a primary role in the pathophysiology of mood and anxiety disorders (MADI), particularly major depression (3-8). Hereafter, we briefly summarize the large and increasing body of evidence that links dysfunction in the glutamate system with MADI.

Evidence linking dysfunction in the glutamate system with mood and anxiety disorders

Clinical evidence: glutamate levels, magnetic resonance and neuroimaging studies

First, changes in glutamate levels have been found in plasma, cerebrospinal fluid (CSF) and brain of MADI patients. A number of studies reported elevated glutamate content, and decreased plasma glutamine/glutamate ratios, in the plasma of depressed patients (9-11). CSF studies found higher glutamate content in depressed patients (12) and lower glutamate in patients with refractory affective disorder (13). Interestingly, although an early study did not find any significant difference in frontal cortex (FC) (14), recent postmortem

studies showed significant increases in glutamate levels in FC and dorsolateral prefrontal cortex (PFC) of depressed and bipolar patients, respectively (15,16).

Second, in vivo proton magnetic resonance spectroscopy studies of MADI have reported consistent alterations in Glx, a combined measure of glutamate, glutamine and GABA (see 17,18 for reviews). Overall, these results suggested abnormalities in glutamate/glutamine/GABA cycling are involved in pathophysiology of MADI, although they do not provide information as to how glutamatergic transmission is changed (4,15,19,20).

Third, a large number of clinical neuroimaging studies of MADI have consistently shown regional volumetric changes in brain areas where glutamate neurons/synapses predominate, such as hippocampus (HPC), cortical regions (including PFC) and amygdala (see for meta-analyses: 21,22). In HPC, volumetric reduction was found particularly associated with repeated depressive episodes (23,24), suggesting correlation with illness duration. Volumetric reduction has been found also for cortical areas, while volumetric enlargement, at least in the early course of illness, has been found for amygdala, suggesting the volume of this stress-related area is dependent on the phase of illness (22).

Preclinical evidence showing structural/functional changes in brain

Although the reasons for volumetric reductions of HPC and PFC in MADI have not yet been clearly identified, it has been proposed that atrophy/remodeling of dendrites is a major factor (Fig. 1) (5-7,25,26). The evidence for this hypothesis comes mostly from experiments with rodents. Numerous studies with stress paradigms and models of depression (for a discussion see: 6,26) have shown that repeated stress exposure, in some cases even a few stress episodes (27), induce atrophy, retraction and simplification of dendritic arbor in CA3 HPC region and medial PFC (28-31). This phenomenon was detected in regions corresponding to those where volumetric reductions have been observed in humans. Dendritic remodeling in rodents was found to be reversible after cessation of stress (32), and antidepressants or mood stabilizers (as well as physical exercise) were shown to partly reverse the structural changes induced by stress (see below). However, the connection between dendritic remodeling and volumetric changes is at present inferential, because it links together complementary evidence obtained from clinical and preclinical studies, and needs further evidence (Fig. 1). Additional factors likely to be primarily involved in brain volumetric changes are

loss of glial cells (particularly in cortical areas) and impairment of neurogenesis (limited to HPC) (33,34).

The role of glucocorticoids and their effects on glutamate release/transmission

A primary role in stress-related dendritic remodeling has been attributed to glucocorticoid hormones, major mediators in the stress response, and to changes in glutamate release/transmission, in turn potently regulated by glucocorticoids. Numerous lines of evidence have shown that both stressors/glucocorticoid treatments acutely increase glutamate release in HPC, PFC/FC, and amygdala (35-40; see 6,26, for a discussion). Interestingly, it has also been shown that enhancement of miniature excitatory postsynaptic currents (EPSCs) in CA1 neurons by corticosterone is mediated by a mineralocorticoid receptor (MR), while acute stress-induced enhancement of depolarization-evoked glutamate release in PFC/FC is mediated by a glucocorticoid receptor (GR) (37,38). Accordingly, it has been argued that stress-induced rise of corticosterone and binding to synaptic MR/GR in turn enhance glutamate release and transmission (3,41).

Therefore, combined evidence has suggested that changes in glutamate release/transmission, elicited by stress/glucocorticoids,

with time induce dendritic remodeling and morphological changes. Remodeling of synapses and neuronal networks is a constant, dynamic and physiological process (42). It can be envisaged that, when glutamate neurotransmission is repeatedly and abnormally overactivated, building up excessive synaptic or extrasynaptic levels of glutamate, physiological mechanisms of synaptic/dendritic remodeling can be transformed into maladaptive changes (7,25,26). However, the effects of chronic stress on glutamate release have been little investigated thus far; future studies are warranted, particularly to investigate how the response to acute stressors is modified by a previous period of chronic stress. The reduction of dendritic arbor by stress corresponds to a reduction of dendritic spines and synaptic boutons, with a shift in PFC from mature mushroom-type to thin- and stubby-type spines (43). Quantitative and qualitative changes in the synapses/circuitry morphology correspond to marked changes in synaptic transmission, likely involved in the 'pathological' behavioral phenotypes observed in stress models. Understanding this maladaptive plasticity may allow to gain insight into the processes whereby adverse environmental factors contribute to the pathogenesis of MADI (3,5), and may open new avenues for the development of novel therapies.

It is noteworthy that monoaminergic-based antidepressants have been repeatedly shown to interfere with the glutamate system, starting with the observation that they modulate the function of the N-methyl-D-aspartate receptor (NMDA-R) for glutamate (44,45). A growing number of studies in recent years have investigated the effects of antidepressants on the glutamate system, unveiling previously unknown effects, likely involved in their therapeutic action (6).

In the following sections we analyze in detail the action of antidepressants on the glutamate system, in particular the release of glutamate and the different classes of glutamate receptors (Fig.2).

Antidepressant agents and the glutamate system

Antidepressants modulate synaptic transmission mediated by AMPA and NMDA receptors

A number of studies have shown that chronic antidepressant treatments of rats reduce excitatory synaptic transmission. Several drugs, including imipramine, citalopram, tianeptine (a neuroprotective antidepressant, serotonin reuptake enhancer), and electroconvulsive shock, attenuated synaptic transmission in FC (46,47). The magnitude of both α–amino-3-hydroxy-methyl-4-isoxazole propionic

acid receptor (AMPA-R) and NMDA-R dependent components of the field potential were reduced, the latter to a larger extent. These findings were in line with previous studies, which showed that antidepressants dampen NMDA-R function (see below). It was also found that imipramine reduced mean frequency and amplitude of spontaneous EPSCs in cortical pyramidal neurons, suggesting reduced presynaptic glutamate release (47).

Antidepressants dampen presynaptic glutamate release

As suggested by the reports showing that chronic antidepressants reduce glutamatergic transmission, a few studies have shown that these drugs actually reduce the presynaptic release of glutamate in HPC and cortical areas. An early study showed that both acute and chronic imipramine and phenelzine significantly reduced glutamate release evoked by depolarization. In PFC, but not striatal slices (48).

In a later study, glutamate/GABA release was measured from HPC purified synaptosomes in superfusion, a technique allowing ex vivo, quantitative detection of endogenous neurotransmitter (3,49). Chronic antidepressant treatments (fluoxetine, desipramine, reboxetine) significantly reduced depolarization-evoked release of glutamate (50). Basal (non-stimulated) glutamate release and

basal/depolarization-dependent GABA release were not changed by drugs. Also acute treatments did not change glutamate/GABA release. Adaptive changes in presynaptic machinery were found altered by antidepressants. In synaptic membranes of drug-treated rats, phosphorylation of α-calcium/calmodulin-dependent protein kinase II (αCaMKII), previously involved in the mechanism of antidepressants (51,52), was markedly decreased. αCaMKII dephosphorylation in turn reduced its binding to syntaxin-1, one of the three SNAREs, and increased syntaxin-1/Munc-18 interaction. As a result, this shift of binding reduces the assembly of syntaxin-1 into the SNARE complex and depolarization-evoked glutamate release (50,53).

A further study showed that acute *in vitro* application of fluoxetine to cerebrocortical synaptosomes reduced 4-aminopyridine–evoked glutamate release, by inhibition of P/Q-type calcium channels (54). However, it is difficult to correlate the *in vitro* drug effect on synaptosomes with the outcome of chronic drug treatments in animals.

These results suggested that reduction of activity-dependent glutamate release by antidepressants could exert a protective action

when the synapse is overactivated by stress-related mechanisms. Regarding the mechanism, it was speculated that chronic antidepressants down-regulate monoamine autoreceptors but also partly affect NA and 5-HT heteroreceptors on glutamatergic terminals, reducing glutamate release (see 55 for a discussion). Moreover, the effects of antidepressants on synaptic glutamate could also be related with a direct modulation of the expression levels of glial-specific glutamate transporters, regulating the neurotransmitter homeostasis (56).

Antidepressants prevent the enhancement of glutamate release induced by acute stress

As addressed above, acute stress and glucocorticoids in rodents consistently induced enhancement of glutamate release in select brain areas. On the other hand, chronic antidepressants reduce presynaptic glutamate release in basal conditions. If stress-induced enhancement of glutamate release/transmission is a causal factor in the induction of synaptic/dendritic remodeling, and antidepressants are able to modulate glutamate release, this effect could be part of the antidepressant action. One of the first studies investigating the effect of psychotropic drugs on the efflux of glutamate induced by

stress showed that diazepam, administered 30 min prior to tail pinch stress, reduced the stress-induced glutamate efflux in HPC and PFC. This effect, explained with the allosteric stimulation of GABA-A receptors, is likely related with the acute anxiolytic action of benzodiazepines (57). However, chronic treatment with lorazepam was shown to reduce glutamate release from PFC slices in superfusion and to increase glutamate release in HPC slices (58), indicating that chronic effects of benzodiazepines can be different from acute effects.

A more recent microdialysis study showed that acute tianeptine or fluoxetine exert different effects on the increase of glutamate efflux induced by acute restraint stress in basolateral amygdala (BLA) and central amygdala (CA) (39). While acute tianeptine completely abolished the stress-induced increase of extracellular glutamate in the BLA, fluoxetine increased basal extracellular glutamate before stress application and potentiated glutamate levels in BLA/CA when stress was applied. Although the acute effect of tianeptine on stress-induced increase of glutamate awaits explanation, the result with fluoxetine provided a potential mechanism for the anxiogenic properties of selective serotonin-reuptake inhibitors, often observed in the initial phase of clinical treatment. The same authors showed

that also acute agomelatine (a new antidepressant with a partially non-monoaminergic mechanism) blocks the stress-induced increase of extracellular glutamate in BLA, CA and HPC (59).

It was recently shown that several chronic antidepressants (fluoxetine, desipramine, venlafaxine, agomelatine) significantly block the enhancement of glutamate release from PFC/FC synaptosomes in superfusion induced by acute footshock (FS) stress (38). Patch-clamp recordings in PFC slices prepared from FS-stressed rats showed that previous chronic desipramine normalized the increase of spontaneous EPSCs amplitude, as well as the marked reduction of paired-pulse facilitation (a measure of glutamate release) and its calcium-dependence. It was also shown that antidepressants did not block the stress-induced rise of circulating corticosterone, which increased glutamate release via GR. Moreover, it was found that FS-stress induced an increase of presynaptic SNARE complexes and of the pool of vesicles ready for release (RRP) in presynaptic membranes (38,60). These findings suggested that the action of antidepressants must be downstream of corticosterone rise and RRP increases. The novelty of this study was that it showed for the first time with the superfusion technique that chronic antidepressants can modify the acute stress response. This newly discovered effect of

antidepressants likely represents a component of the therapeutic effect, particularly the anxiolytic action. Further research is warranted to understand whether other classes of drugs share this long-term action of antidepressants, and whether compounds reducing stress-induced glutamate release in cortical areas could be efficient anxiolytic drugs.

Antidepressants and mood stabilizers reverse stress-induced reduction in dendritic arborization

If antidepressants reduce the enhancement of glutamate release/transmission induced by stressors, they should also be able to prevent or reverse the dendritic remodeling induced by repeated stress. Indeed, a number of studies have shown that antidepressants or mood stabilizers may reverse the structural changes observed in stress/depression models. Both tianeptine and lithium prevented dendritic remodeling caused by chronic restraint stress in HPC (29,61). A different animal model of depression, the olfactory bulbectomy, displays reduced spine density in CA1, CA3 and DG HPC areas; chronic amytriptiline reversed the spine reduction in all three areas, while mianserin reversed the reduction in DG only (62). More recently, subchronic (6 days) administration of desipramine

was shown to rescue the reduction of spines in HPC and the escape deficit in the Learned Helplessness (LH) model of depression (63). Moreover, different antidepressants have been shown to reverse the reduction in dendritic arborization and spine density induced by chronic mild stress (64).

Antidepressants regulate glutamate receptors

Glutamate released in the synaptic cleft can bind ionotropic postsynaptic glutamate receptors, including AMPA-R, NMDA-R and kainate receptors, or metabotropic glutamate receptors (mGluRs), localized at both pre- and postsynaptic sites. A number of studies have consistently shown that chronic antidepressants selectively regulate glutamate receptors expression and function (Tab. 1).

In particular, it was demonstrated that antidepressants differentially modulate ionotropic receptors, leading to a reduction of NMDA-R function and a relative potentiation of AMPA-R-mediated transmission. Different antidepressants were shown to reduce radioligand binding to rat cortical NMDA-R (44,65) and decrease both mRNAs and protein expression levels of NMDA-R subunits (55,66). It was also shown that early-life stress increased the expression of GluN1 subunit of NMDA-R in HPC of the Flinders rat model of

depression, while chronic mild stress increased both transcriptional and protein expression of NMDA-R subunits in HPC. Chronic treatment with respectively escitalopram and duloxetine was able to normalize these alterations (67,68).

On the other hand, antidepressants also exert a positive regulation of AMPA-R expression and phosphorylation. Chronic antidepressants increased immunoreactivity of AMPA-R subunits GluA1 and GluA2/3 in both rat HPC and cortical areas (69-71): fluoxetine increased GluA1 phosphorylation at Ser845 (associated with increased GluA1 insertion), and imipramine increased synaptic expression of GluA1 in HPC (72,73). Intriguingly, it was also recently demonstrated that tianeptine, in addition to increasing GluA1 synaptic expression, also stabilizes membrane exposure of AMPA-R, by reducing their surface diffusion, favoring long-term synaptic plasticity (74).

Moreover, chronic antidepressants also induce specific changes in the expression and function of individual mGluR subtypes (75). mGluRs are separated into three groups based on sequence homology, second messenger coupling, and agonist selectivity. Group I mGluRs (mGlu1/mGlu5) are postsynaptic excitatory receptors positively coupled with phospholipase C. Group II mGluRs (mGlu2/mGlu3) are autoreceptors localized mainly at extrasynaptic

sites on glutamatergic neurons and modulate glutamate release (76). Group III mGluRs (mGlu4/mGlu6/mGlu7/mGlu8) are heterogeneous: mGlu4, mGlu7 and mGlu8 are mainly presynaptic and function as autoreceptors to inhibit glutamate or GABA release, while mGlu6 is a postsynaptic receptor mediating synaptic transmission at retinal cells. Repeated electroconvulsive therapy and prolonged imipramine treatment were shown to increase significantly the expression of group I mGluRs in HPC (77). It was argued that upregulation of group I mGluRs may reflect a compensatory mechanism caused by receptor subsensitivity consequent to antidepressant treatment. Moreover, chronic imipramine increased the expression of mGlu2/3 subunits in the rat HPC, cortex, caudate nucleus and nucleus accumbens (78).

Finally, mGlu7 (but not mGlu4) expression levels were decreased by citalopram, but not imipramine, both in rat HPC and cortex (79). It was recently found that chronic fluoxetine did not change mGlu4 expression in HPC of control rats, while restored mGlu4 levels reduced by early-life stress. On the other hand, mGlu7 and mGlu8 were up-regulated by fluoxetine in control rats but not in stressed rats (80).

Targeting glutamate ionotropic receptors in the treatment of mood disorders

Given the role of impaired regulation of glutamatergic neurotransmission in the pathophysiology of MADI, and the long-term modulation of synaptic function induced by traditional antidepressants, growing interest addressed the development of drugs directly targeted on glutamatergic system. This new pharmacological approach mainly aims at shortening the onset of therapeutic effects, reducing the number of non-responders, and avoiding the side effects of monoaminergic antidepressants. Indeed, early studies showed that NMDA-R antagonists exert antidepressant-like action, paving the way for more recent studies on this class of drugs. Moreover, positive modulators of AMPA-R have shown antidepressant effects. Also a number of mGluR modulators were proposed as alternative approach for regulation of glutamatergic function.

A number of recent, exciting studies reported rapid antidepressant action, both in preclinical and clinical studies, of low doses of high-affinity non-competitive NMDA-R antagonists (particularly the dissociative anesthetic ketamine). It has been shown that a single subanesthetic dose of ketamine induces rapid (within hours) and

sustained (up to 1 week) antidepressant efficacy in treatment-resistant patients, although the actual response to ketamine might be longer (81-83). These findings, particularly robustness, rapidity and durability of the antidepressant effect, were unanticipated. Ketamine appears to target directly the core depressive symptoms such as sad mood, suicidality and helplessness, rather than inducing a nonspecific mood-elevating effect.

The drug has also been shown antidepressant properties in different rodent models of depression. It rapidly reversed depressive-like behavior in two well-established models, the forced swim test (FST) and LH, as well as in a model of anxiety, the novelty suppressed feeding test (84), and reversed anhedonic/anxiogenic behaviors and functional/morphological alterations induced by chronic unpredictable stress (CUS) (85). However, the long-lasting effect of ketamine was not replicated in a different study, where a higher dose (50-160 mg/kg) was used (86).

Although the mechanism has not been completely elucidated, it was consistently found that ketamine rapidly activates the mammalian target of rapamycin (mTOR) pathway, inducing a sustained increase in the expression levels of synaptic proteins and in the number of excitatory spine synapses in PFC. This suggests that the behavioral

effects of ketamine could be related with changes in synaptic plasticity and synaptogenesis (84,85). Somewhat comparable effects have been described after chronic treatments with traditional antidepressants; it is not know at present whether ketamine induces these restoring effects by-passing the monoamine-mediated effects or by a completely different mechanism.

Unfortunately, the adverse effects of ketamine, especially psychotomimetic consequences and cognitive impairment, limit a wide clinical use of this drug (83). Therefore, a number of other NMDA-R modulators (competitive NMDA-R antagonists, partial antagonists of glycine site, high- and low-affinity non-competitive NMDA-R antagonists) are currently under scrutiny as potential antidepressants. An emerging research approach is directed to the development of compounds selectively acting on specific NMDA-R subunits, particularly GluN2B. Ro25-6981, a GluN2B selective antagonist, showed antidepressant activity similar to ketamine in preclinical models, inducing significant dose-dependent reduction of immobility in the FST in mice (87), and rapidly ameliorating CUS-induced anhedonic behaviors in rats (86).

A complementary pharmacological approach consists in targeting AMPA-R to modulate glutamatergic transmission. Interestingly, it was

shown that the antidepressant action of ketamine, but not of a different NMDA antagonist (88), requires rapid AMPA-R activation (87), and that an increase in the AMPA-R/NMDA-R ratio plays a key role in the induction of antidepressant effects (89). It may appear counterintuitive that AMPA potentiators have antidepressant effect, knowing that antidepressants have been shown to dampen glutamate release (see above). However, AMPA potentiators work by increasing the response of AMPA-R, which is independent on the actual level of glutamate release. Several classes of AMPA-R potentiators, including nootropic agents and ampakines, have shown antidepressant efficacy in preclinical studies (90,91), and a number of compounds are currently under clinical development.

Targeting metabotropic glutamate receptors modulators in the treatment of mood disorders

Targeting metabotropic glutamate receptors represents an interesting alternative for modulation of glutamatergic transmission. Indeed, signaling via mGluRs is slower and longer-lasting than for ionotropic glutamate receptors, allowing a fine tuning of the cellular response to glutamate signaling. Accumulating evidence suggested anxiolytic and antidepressant effects of selective agents acting on mGluRs

(92,93). Group I mGluR, especially mGlu5, selective antagonists and mGlu7 antagonists showed antidepressant efficacy in preclinical studies (92,93) and two clinical studies of mGlu5 antagonists have been concluded (http://www.clinicaltrials.gov/ct2/show/record/NCT00809562?term=RO4917523&rank=9, http://www.clinicaltrials.gov/ct2/show/NCT01145755?term=AZD2066&rank=9). Intriguingly, both selective mGlu2/3 receptor agonists and antagonists exhibit antidepressant-like activity. Agonists of presynaptic mGlu2 receptors likely act by reducing excess of glutamate release. On the other hand, mGlu2/3 antagonists may increase synaptic levels of glutamate, potentiating AMPA-R-mediated transmission, and firing rates of 5-HT and DA neurons, whereby increasing extracellular levels of monoamines. Moreover, it was shown that, as for ketamine, the behavioral effects of selective mGlu2/3 antagonists are dependent on mTOR signaling (94, 95).

Taken together, these results suggest that modulation of mGluRs may be of value in the treatment of depression.

Conclusions and future perspectives

Recent times have seen a strong revival of research on the involvement of glutamate system in MADI and of investigation of glutamate-based therapeutic approaches, although many unanswered questions remain on how changes in glutamate-related processes mediate pathophysiology or drug mechanisms. There are currently several lines of preclinical/clinical research, investigating the action of molecules that directly target different sites of regulation of the glutamate synapse, including antagonists of NMDA-R, positive modulators of AMPA-R and mGluR agonists/antagonists. Extensive cellular/molecular investigation has recently shown that all major sites of regulation of the glutamate synapse, including presynaptic release machinery, postsynaptic ionotropic receptors, pre- and postsynaptic metabotropic receptors, glutamate reuptake and glutamate/glutamine recycling mechanism, represent at the same time targets of stress and potential targets for pharmacological modulation (3). It is likely that, as research on pathology-related changes at glutamate synapses and circuitry will progress, new targets and new investigational drugs will appear on the stage. In this regard, while it is known that acute stress in rodents rapidly enhances glutamate release/transmission in select areas, like HPC

and PFC, the outcome of repeated stress seems to reverse this situation in the same areas, inducing dendritic retraction, loss of spines/synapses and maladaptive remodelling of the glutamate circuitry (see Fig. 1). In some cases, this structural remodeling has been shown associated with concomitant impairment of cognitive functions, such as spatial working memory and attentional set shifting task, or with depressive-like behavior (31,63,96). The cellular/molecular adaptive changes involved in the transition between the effects of acute and repeated stress on synapses and circuitry could be crucial targets for therapies that are able to block or reverse maladaptive changes. Among the candidates available, a likely mediator of key adaptive/maladaptive changes induced by either antidepressants/physical exercise or stress, is Brain Derived Neurotrophic Factor (BDNF) (7,25,97,98), in particular its regulation of expression and synaptic trafficking (99,100). Current studies are investigating the effects of stress and restorative treatments on select splice variants of BDNF transcripts and their role in structural/functional synaptic plasticity.

A further candidate target is the presynaptic machinery of glutamate release, which is regulated by traditional antidepressants in the acute stress response (see above). Previous findings have shown that

these drugs do not block the stress-induced rise of corticosterone levels, or the stress/glucocorticoid-induced increase of glutamate RRP in synaptic terminals, suggesting a modulation of release machinery (38,60). The stabilizing action of antidepressants on the stress-induced enhancement of glutamate release may suggest a new putative line of drug development, directly targeted at the molecular mechanisms regulating glutamate release, which may provide a means of attenuating exaggerated or inadequate stress response in MADI. Current studies are investigating: (1) whether blockade of stress-induced glutamate release is exclusive to antidepressants; (2) the preventing effect of drugs on stress-induced glutamate release as a functional test to assay molecules with antidepressant/anxiolytic action; and (3) what is the site of action of antidepressants in this mechanism.

Undoubtedly, the employment of investigational drugs acting directly on the glutamate system has opened new avenues in the development of drugs and therapies for neuropsychiatric disorders. In this perspective, the rapid action of glutamate receptor antagonists is certainly a fascinating and promising feature for second-generation antidepressants, allowing for rapid clinical effects with possibly lower rates of remission.

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Figure legends

Figure 1

Structural/functional changes in the glutamate system linked to pathophysiology of mood and anxiety disorders and to the action of antidepressants.

Sequelae of events putatively involved in adaptive or maladaptive neuroplasticity changes related to the effects of stress and psychiatric pathophysiology, or of restorative factors (antidepressant treatments, physical exercise).

Preclinical studies showed that inadequate or excessive stress response, through the action of glucocorticoids and other neurochemical/neuroendocrine mediators, may induce (2) abnormal enhancement of glutamate release and excitatory transmission in select brain areas, including amygdala, hippocampus and prefrontal cortex. This in turn induces (3) atrophy and remodeling of dendritic arbor at various locations in these areas (seemingly the opposite in amygdala in a first phase), with loss of dendritic spines and synapses. In turn, dendritic/circuitry remodeling is envisaged as a causal factor for volumetric changes observed with MRI in depressed

subjects (4), together with other factors, including glial loss and impairment of neurogenesis (in hippocampus).

The evidence that stress and consequent changes in excitatory transmission cause dendritic remodeling comes mostly from rodent studies, while volumetric changes have been observed in humans. Therefore, the connection is at present inferential and needs additional evidence. The changes induced by stress paradigms in rodents are reversible with cessation of stress. Chronic antidepressant treatments and voluntary physical exercise are able to prevent or reverse partly the effects of stress on glutamate release and neurotransmission and on dendritic remodelling (see text). Adapted and modified from ref. 26. Figure was produced using Servier Medical Art (www.servier.com).

Figure 2

Sites of action (targets) of antidepressants in the glutamate tripartite synapse.

Growing evidence showed a key role of the modulation of glutamatergic neurotrasmission in the action of antidepressant drugs. Several sites/mechanisms of regulation of the glutamate synapse have been shown to be targets of antidepressants: (1) presynaptic

release of glutamate; (2) postsynaptic ionotropic receptors for glutamate (NMDA and AMPA receptors); (3) metabotropic glutamate receptors; (4) glial-specific glutamate transporters. See text for details. Adapted from ref. 3.

Figure 1

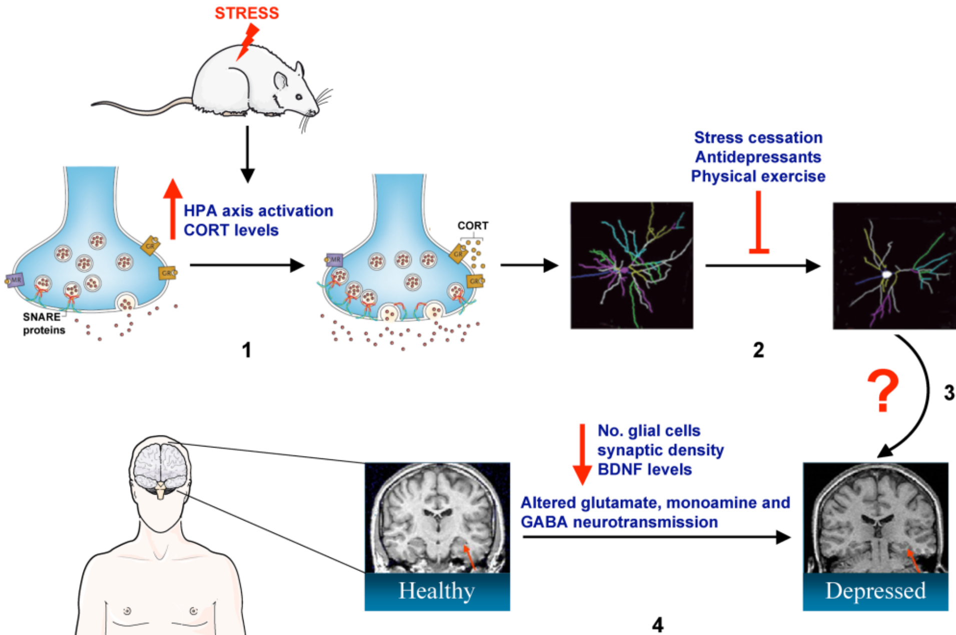

Figure 2

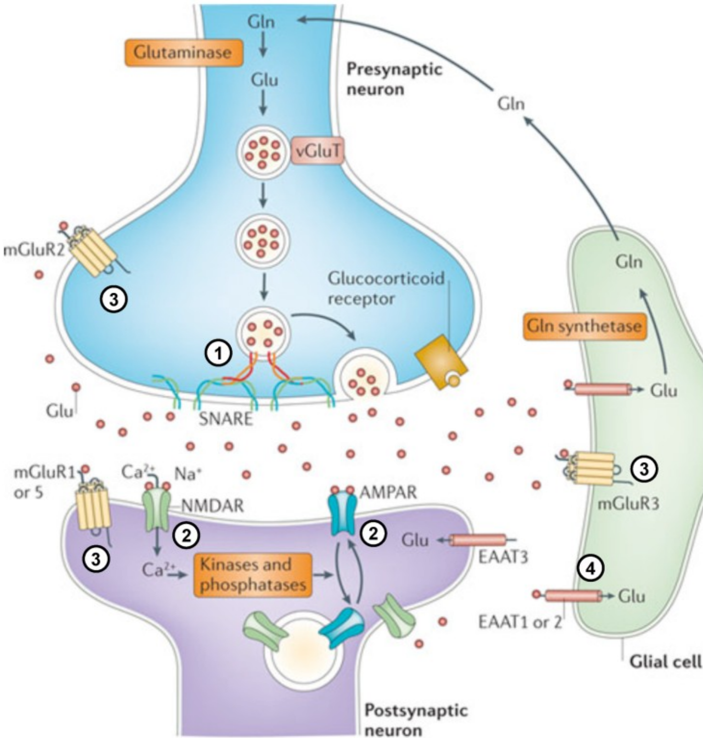

Table 1

Effects of antidepressant treatments on glutamatergic receptors function and expression.

Brain region	Antidepressant	Glutamate receptor (subunit)	Change	Reference
Cerebral cortex	Imipramine and Citalopram	NMDA-R	⇓ radioligand binding	Paul et al., 1994
Cerebral cortex	Imipramine and Citalopram	NMDA-R	⇓ radioligand binding	Skolnick et al., 1996
Cerebral cortex	Imipramine	NMDA-R	⇓ radioligand binding	Nowak et al., 1998
Hippocampus	Fluoxetine and Reboxetine	GluN1	⇓ protein expression	Pittaluga et al. 2007
Hippocampus	Escitalopram	GluN1 GluA1	⇓ protein expression	Ryan et al., 2009
Ventral hippocampus	Duloxetine	GluN1 GluN2A	⇑ protein expression	Calabrese et al., 2012
Cerebral cortex	Fluoxetine	GluN2A, GluA1, GluA2	⇑ protein expression	Ampuero et al., 2010
Hippocampus	Desipramine and Paroxetine	GluA1, GluA2/3	⇑ protein expression	Martinez-Turrillas et al., 2002
Hippocampus	Desipramine and Paroxetine	GluA1, GluA2/3	⇑ protein expression	Martinez-Turrillas et al., 2005
Prefrontal cortex	Fluoxetine Desipramine Reboxetine Reboxetine	GluA4 GluA3 GluA2/4 GluA3/5	⇑ protein expression ⇓ protein expression ⇑ protein expression ⇓ protein expression	Barbon et al., 2006
Hippocampus	Fluoxetine Fluoxetine Desipramine Reboxetine	GluA2/6 GluA5 GluA3 GluA2/4/6	⇓ protein expression ⇑ protein expression ⇓ protein expression ⇑ protein expression	Barbon et al., 2006
Nucleus accumbens, dorsal striatum and	Maprotiline	GluA1, GluA2/3	⇑ protein expression	Tan et al., 2006

hippocampus				
Hippocampus, cerebral cortex and striatum	Fluoxetine	GluA1	⇑ phosphorylation at Ser-831 and Ser-845	Svenningsson et al., 2002
Hippocampus	Ketamine	GluA1	⇓ phosphorylation at Ser-845	Maeng et al., 2008
Hippocampus	Tianeptine	GluA1	⇑ phosphorylation at Ser-831 and Ser-845	Szegedi et al., 2011
Hippocampal neurons	Tianeptine	GluA1	⇓ surface diffusion	Zhang et al., 2012
Hippocampus	Imipramine	mGlu1a	⇑ protein expression	Bajkowska et al., 1999
Hippocampus	Imipramine	mGlu5a	⇑ protein expression	Smialowska et al., 2002
Hippocampus	Imipramine	mGlu2/3 mGlu4, mGlu5	⇑ protein expression = protein expression	Matrisciano et al., 2002
Frontal cortex and hippocampus	Citalopram	mGlu7 mGlu4	⇓ protein expression = protein expression	Wieronska et al., 2007
Hippocampus	Fluoxetine	mGlu7, mGlu8	⇑ protein expression	O’Connor et al., 2012

CHAPTER II

Journal of Neuroscience (submitted)

Corticosterone rapidly increases the readily releasable pool of glutamate vesicles in synaptic terminals of prefrontal and frontal cortex by acting on multiple local receptors

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Keywords

Stress, Corticosterone, Glutamate release, Presynaptic mechanisms, Mood disorder

ABSTRACT

Converging lines of evidence have shown that stress and glucocorticoids alter glutamatergic transmission, suggesting this is a core pathophysiological feature in neuropsychiatric disorders. We have previously demonstrated that acute stress rapidly increases presynaptic glutamate release in prefrontal and frontal cortex (PFC/FC) via glucocorticoid receptor (GR) and accumulation of presynaptic SNARE complex.

In the present work we compared the *ex vivo* effects of acute stress with those of *in vitro* application of corticosterone, to analyze whether the acute effect of stress on glutamatergic transmission is mediated by a synaptic action of corticosterone. We found that *in vitro* application of corticosterone to brain slices from naïve rats does not alter excitatory synaptic transmission in medial PFC. Then, we measured glutamate release evoked by KCl-depolarization and by hypertonic sucrose (to measure readily releasable pool [RRP]), from purified synaptosomes in superfusion. We found that acute stress increases both depolarization-dependent release and RRP, while *in vitro* application of corticosterone only increases the RRP, an effect dependent on GR and mineralocorticoid receptor (MR). By using total internal reflection fluorescence microscopy, a technique allowing the

study of events occurring just below the plasma membrane, we confirmed that corticosterone induces a rapid mobilization of synaptic vesicles in the vicinity of the presynaptic membrane, again dependent on both GR and MR. Finally, we found that both stress and corticosterone *in vitro* selectively increase the phosphorylation at site 1 of synapsin I in presynaptic membranes.

The present results suggest that corticosterone mediates the enhancement of glutamate release induced by acute stress in PFC/FC, and the hormone is necessary but not sufficient for this effect.

INTRODUCTION

In the last several years, convergent clinical and preclinical evidence supported a key role for dysfunctional glutamatergic transmission and synapses in the pathophysiology of mood and anxiety disorders (MADI) (reviewed in McEwen, 2005; Sanacora et al., 2012). Interestingly, it was shown that stress and its hormonal mediators, cortisol in humans and corticosterone (CORT) in rodents, modulate glutamatergic transmission in different brain areas, including hippocampus, amygdala and prefrontal cortex (PFC), effects that have been associated with MADI onset and exacerbation (as a review, see Prager and Johnson, 2009; Musazzi et al., 2011; Popoli et al., 2012).

Corticosteroids bind to mineralocorticoid and glucocorticoid receptors (MR and GR, respectively), classically shown as transcriptional factors mediating delayed genomic effects. However, recent studies showed that CORT also produces rapid non-genomic effects on neuronal excitability and function through the activation of putatively membrane-associated GR/MR. Indeed, it has been demonstrated that acute *in vitro* CORT application on hippocampal slices enhances the frequency of miniature excitatory postsynaptic potentials by presynaptic non-genomic MR-dependent mechanisms, suggesting

an increase in the release probability of glutamate (Karst et al., 2005, 2010). In hippocampus, CORT exerts also non-genomic postsynaptic effects, potentiating the excitability of postsynaptic neurons through MR activation, inhibiting repolarizing potassium I_A-currents (Olijslagers et al., 2008) and facilitating the induction of synaptic plasticity, through changes in α-amino-3-hydroxyl-5-metyl-4-isoxazole propionic acid (AMPA) receptors surface trafficking (Groc et al., 2008).

We have recently demonstrated that acute footshock (FS)-stress induces a rapid increase of depolarization-evoked glutamate release from prefrontal and frontal cortex (PFC/FC) synaptic terminals (synaptosomes) (Musazzi et al., 2010). This effect was dependent on the activation of GR and rapid accumulation of SNARE complexes in presynaptic membranes, suggesting an increase of the pool of synaptic vesicles (SV) ready for release (RRP).

Main aim of the present work was to compare the *ex vivo* effect of acute stress with that of *in vitro* application of CORT on purified synaptosomes, to analyze whether the acute effect of stress on glutamatergic transmission was mediated by a non-genomic, synaptic action of CORT. First, we investigated the effect of *in vitro* application of CORT on excitatory synaptic transmission in medial

PFC (mPFC), by using intracellular recordings in brain slices from naïve rats. Second, to assess whether CORT modifies the size of RRP, we measured glutamate release evoked by KCl-depolarization and by hypertonic sucrose, by using purified synaptosomes in superfusion. Third, we investigated whether CORT increases SV mobilization by using total internal reflection fluorescence microscopy (TIRFM), a technique that allows the study of events occurring in a 100 nm-interval below the plasma membrane (Groves et al., 2008). Finally, we studied if acute stress and CORT modulated the phosphorylation of synapsin I, a presynaptic protein involved in SV mobilization and in docking, fusion and recycling at active zones (Cesca et al., 2010). Our results show that CORT rapidly increases the size of RRP but not depolarization-dependent glutamate release in PFC/FC, that this effect is mediated by both GR and MR located at synaptic terminals and is accompanied by selective phosphorylation at site 1 of synapsin I in presynaptic membranes.

MATHERIALS AND METHODS

Animals

All experimental procedures involving animals were performed in accordance with the European Community Council Directive 86/609/EEC and were approved by Italian legislation on animal experimentation (Decreto Legislativo 116/1992). Sprague-Dawley male rats were used.

Electrophysiology

Intracellular recordings were performed essentially as previously reported (Musazzi et al., 2010). 175-200g rats were anesthetized by intraperitoneal injection of 50 mg thiopental and perfused with an ice-cold recording solution ($NaHCO_3$ buffered saline bubbled with 95% O_2 5% CO_2 and containing, in mM: 119 NaCl, 2.5 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 11 D-glucose). After decapitation, brains were quickly removed and transferred to an ice-cold cutting solution (a modified version of the recording solution, with 1 mM $CaCl_2$ and 3 mM $MgSO_4$). mPFC was cut into 450 µm tick coronal slices with a Vibratome. Then slices were incubated for 1–6 h at room temperature (20–22°C) in recording solution before to be transferred in the recording chamber. Layer III pyramidal neurons of

mPFC were impaled with sharp micro-pipette electrodes (resistance 180-200 MΩ) containing Cs-methanesulfonate 2 M and QX-314 10 mM for intracellular recording of excitatory post-synaptic potentials (EPSPs) evoked by extracellular stimulation of layer V (stimulation period: T_{stim}= 10 s). Bipolar stimulation was applied on the V layer with a pair of tungsten electrodes (R=10 MΩ, inter-electrode distance 300μm), with biphasic pulses of length 200 μs and constant amplitude (15−40 pA). Cells were recorded if showing a stable resting potential of at least -80 mV. Constant current was provided to the cell (max 1nA) for stabilizing the resting potential if needed. The amplitude of the EPSP was measured as peak-to-baseline difference, averaged in groups of six (one sample for each minute of recording), and divided by the first value of the series (equal to15.164 ±0.391 mV, mean plus standard deviation on the set). The paired-pulse ratio was computed as the amplitude ratio between the first and the second EPSPs (measured as peak-to-baseline difference) and then averaged in groups of six (one sample for each minute of recordings).

Footshock stress procedure

The footshock (FS)-stress protocol was performed on 275-300 g male rats essentially as previously reported (Musazzi et al., 2010) (40-min FS-stress; 0.8 mA, 20 min total of actual shock with random intershock length between 2–8 sec). The FS-stress box was connected to a scrambler controller (LE 100-26, Panlab) that delivers intermittent shocks to the metallic floor. Sham-stressed rats (controls) were kept in the stress apparatus without delivering of shocks. Animals were sacrificed by decapitation. Serum was prepared from trunk blood by centrifugation and stored at -80°C. Measurement of serum CORT levels was performed by a commercial kit (Corticosterone EIA kit, Enzo Life Science).

Preparation of purified synaptosomes

The whole frontal lobe, referred to as PFC/FC, was quickly excised on ice as previously reported (Musazzi et al., 2010).

Purified synaptic terminals (synaptosomes) were prepared by centrifugation on Percoll gradients (Dunkley et al., 1986), with minor modifications (Bonanno et al., 2005) from fresh PFC/FC. Purity of synaptosomes and other subcellular fractions were evaluated by electron microscopy and by measuring subcellular distribution of

protein markers (not shown), as previously reported (Barbiero et al., 2007). For glutamate release experiments and TIRFM experiments, synaptosomes were resuspended in physiological medium with the following composition: 140 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM glucose, 10mM HEPES, pH 7.2–7.4. For western blotting experiments, synaptosomes were resuspended in lysis buffer: 120 mM NaCl, 20 mM HEPES pH 7.4, 0.1 mM EGTA, 0.1 mM DTT, containing 20 mM NaF, 5 mM Na_2PO_4, 1 mM Na_2VO_4, and 2 mg/ml of protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy). The synaptic membrane fraction was prepared by centrifugation as previously reported (Musazzi et al., 2010). The triton insoluble fraction (TIF) was prepared as reported in Gardoni et al., 2006.

Glutamate and GABA release experiments

Synaptosomes were incubated at 37° C for 15 min in the presence of 0.02 µM [3H]D-Asp (a non-metabolizable analogue of glutamate (Glu) used to label the synaptosomal Glu releasing pools) or without label (experiments of endogenous neurotransmitter release). Aliquots of the synaptosomal suspension were layered on microporous filters placed at the bottom of a set of parallel superfusion chambers

maintained at 37° C (Raiteri et al., 2002). Superfusion was then started with standard medium at a rate of 0.5 mL/min and continued for 48 min. After 36 min of superfusion to equilibrate the system, samples were collected as follows: two 3-min samples (t = 36–39 and 45–48 min; basal efflux) before and after one 6-min sample (t = 39–45 min; stimulus-evoked release). When depolarization-evoked release was assayed, stimulation with a 90-s pulse of 15 mM KCl was applied at t = 39 min. In the experiments measuring the RRP, synaptosomes were exposed to a 15 s pulse of 250-500 mM sucrose at t = 39 min. When [3H]D-Asp was used collected samples and superfused synaptosomes were counted for radioactivity. Radioactivity was determined in each sample collected and in the superfused filters by liquid scintillation counting. 3H released in each sample was calculated as fractional rate X 100 (percentage of the total synaptosomal neurotransmitter content at the beginning of the respective sample collection). Endogenous Glu and GABA were measured by HPLC analysis following pre-column derivatization with o-phthalaldehyde and separation on a C18 reverse-phase chromatographic column (10 X 4.6 mm, 3 µm; at 30° C; Chrompack, Middleburg, the Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm; Raiteri

et al. 2000). Homoserine was used as an internal standard. The released endogenous Glu and GABA were expressed as pmol/mg of synaptosomal protein. The stimulus-evoked neurotransmitter overflow was estimated by subtracting the transmitter content of the two 3-min samples representing the basal efflux from the content of the 6-min sample collected during and after the stimulating pulse (total stimulus-evoked neurotransmitter release).

For the experiments of incubation *in vitro* with CORT, synaptosomes were perfused with CORT during the pulse of 15 mM KCl or 250-500 mM sucrose. When selective GR MR antagonists where used to counteract the action of CORT, 100 nM SPIR (selective MR antagonist) and 500 nM RU486 (GR antagonist) were added during CORT incubation (Karst et al., 2005; 2010; Groc et al., 2008).

Immunofluorescence and Total Internal Reflection Fluorescence Microscopy

For immunofluorescence experiments, 150 µg of freshly purified synaptosomes from PFC/FC were layered on coverslips for 2 hours at room temperature. Then, synaptosomes were incubated with the lipophilic dye 4 µM FM1-43 FX 4 µM (fixable analog of FM1-43, Life Technologies) in the presence of 25 mM KCl for 100 sec, for vesicle

loading, and then washed in physiological buffer at least for 10 minutes to remove the staining of plasma membranes (Serulle et al., 2007). Synaptosomes were then fixed in glutaraldehyde 0.2% for 15 minutes at room temperature. Fixed synaptosomes were incubated with anti L-glutamate (Millipore) 1:200 followed by Alexa Fluor 555 goat anti-rabbit IgG (Life Technologies) and imaged by TIRFM, to determinate the amount of FM1-43 FX-positive glutamatergic structures.

Synaptosomes were imaged under TIRF illumination by means of an AxioObserver Z1 microscope (Zeiss, Jena, Germany) (Perego et al, 2012; D'Amico et al., 2010). Synaptosomes were identified by fluorescence using a 100 X oil immersion objective (NA 1.45 Alpha-Plan, Zeiss), and TIRFM was performed using a 100 mW multiline argon-ion laser introduced into the excitation light path (filters 488/10 nm for FM1-43 or 514/10 nm for glutamate-Alexa 555) through the TIRF-slider (Zeiss). The band widths of the emissions were 500–550 nm (FM1-43) and 550–600 nm (Alexa 555). Digital images (512 × 512 pixels) were captured on a cooled Retiga SRV CCD camera.

Total Internal Reflection Fluorescence Microscopy on live synaptosomes

For TIRFM experiments on live synaptosomes, 50 µg of freshly purified synaptosomes from PFC/FC were layered on coverslips for 2 hours at room temperature. Then, synaptosomes were incubated with 4 µM FM1-43 in the presence of 25 mM KCl for 100 sec, as above. After extensive washing, synaptosomes were imaged under TIRF illumination for 10 minutes in the presence of 100 nM, 1 µM, 10 µM CORT or DMSO 0.1%. Imaging was carried out at 4 frames/min during the first 5 minutes and 1 frame/min during the next 5 minutes of incubation. When RU486 and SPIR were used, synaptosomes were pre-incubated for 5 minutes with either 50 µM RU486 or 50 µM spironolactone in 0.1% DMSO before 10 µM CORT treatment (Karst et al., 2005; Zhou et al., 2011). During the pre-incubation with antagonists, TIRFM recordings were routinely carried out at 1 frame/min.

Image analysis on Total Internal Reflection Fluorescence Microscopy experiments

TIRF images were analyzed using Image-Pro Plus Analyser Image Software (Media Cybernetics, Bethesda, MD, USA). A set of

automated image processing macro/subroutines was developed based on existing algorithms of the Image-Pro Plus Analyser software (Gaussian filtering, Nearest neighboring deconvolution) and, lastly, images were background-subtracted by using the averaged value within a user-defined background region. The resulting corrected images were then analyzed using a plugin custom written for selection and quantification of fluorescent spots according to their shapes, size and intensity. The following criteria were used to include individual structures: (1) the mean diameter was 0.5 mm, (2) the minimal pixel intensity was 30% above the local background, and (3) the roundness score was 0.8-3.5.

In immunofluorescence experiments, data acquired using multicolor TIRF, once selected, were processed to measure colocalization of individual structures.

In TIRFM experiments on live synaptosomes, images were analyzed as reported above. The number of fluorescent spots in the TIRF field in each frame was normalized on the number of spots at t = 0 minute and expressed as percentage vs t= 0 minute.

Western blotting

To measure expression and phosphorylation levels of synapsin I, synaptosomes were resuspended in physiological medium containing 10 µM CORT or 0.1% DMSO and incubated 10 minutes at 37° C under gentle shaking. For the experiments with the antagonists SPIR and RU486 were added 5 minutes before and during CORT incubation. After incubation, synaptosomes were centrifugated at 4° C and resuspended in cold lysis buffer. The synaptic membrane fraction was prepared by centrifugation as described above.

Western blotting analysis was carried out as previously described (Musazzi et al. 2010) by incubating PVDF membranes, containing electrophoresed and blotted proteins from either synaptic terminals or presynaptic membranes, with monoclonal antibodies for synapsin I 1:2000 (Synaptic System, Gottingen, Germany), P-synapsin I site 7 (Ser553) 1:1000 (Abcam), b-actin 1:20000 (Sigma-Aldrich), and polyclonal antibodies for P-synapsin I site 1 (Ser9) 1:1000 (Cell Signaling), P-synapsin I site 3 (Ser603) 1:1000 (Abnova), P-synapsin I site 4,5 (Ser62-67) 1:500 (Merck Millipore), P-synapsin I site 6 (ser549) 1:1000 (Merck Millipore), GR 1:500 (SantaCruz Biotechnologies), MR 1:500 (Santa Cruz Biotechnologies). After incubation with peroxidase-conjugated secondary antibodies, protein

bands were detected with ECL^{TM} (Genespin). All protein bands used were within a linear range, and normalized for b-actin level in the same membrane.

Drugs

[3H]D-Aspartate ([3H]D-Asp, specific activity: 16.3 Ci/mmol), was purchased from Amersham (Buckinghamshire, UK). CORT, SPIR, RU486 were from Sigma-Aldrich.

Statistical analysis

One- way analysis of variance (ANOVA) followed by the Newman-Keuls test (when more than two experimental groups were analyzed) or the unpaired Student's t test (when two experimental groups were analyzed) were used for the analysis of release experiments.

One- way analysis of variance (ANOVA) followed by the Newman-Keuls test was used for the analysis of the AUC values obtained from TIRFM experiments. One- way analysis of variance (ANOVA) followed by the Newman- Keuls test or the unpaired Student's t test were used for the analysis of Western blotting experiments.

Statistical analysis of the data was carried out using GraphPad Prism4 (GraphPad Software Inc., USA).

RESULTS

In vitro application of corticosterone does not affect glutamate synaptic transmission in slices from medial prefrontal cortex

In order to understand if acute *in vitro* application of CORT was able to modify synaptic transmission in mPFC, we incubated mPFC slices with 10 μM CORT and recorded the excitatory post-synaptic potentials (EPSPs) in pyramidal neurons of layer III of mPFC evoked by extracellular stimulation of layer V. We found that application of CORT for up to 20 min did not alter EPSPs amplitude nor affect resting potential (Fig. 1A). To evaluate the effect of CORT on a form of short–term synaptic plasticity that is used to monitor changes in glutamate release, we measured the paired pulse facilitation (PPF), by recording EPSPs evoked by pairs of stimuli applied at increasing interpulse intervals (Fig. 1B). The analysis of the paired-pulse ratio (PPR) of the second to the first eEPSP revealed that incubation with CORT did not induce synaptic facilitation.

Considering that the mechanisms underlying neural facilitation are exclusively presynaptic, these experiments suggest that CORT did not affect presynaptic release evoked by electrical stimulation.

Acute stress and corticosterone *in vitro* increase the readily releasable pool of glutamate from prefrontal and frontal cortex synaptosomes but only stress enhances depolarization-dependent release of glutamate.

Our previous studies showed that acute FS-stress, via rapid increase in circulating CORT level, enhances depolarization-evoked glutamate release from PFC/FC synaptosomes. This effect was shown to be dependent on GR activation and accompanied by SNARE protein complex accumulation in presynaptic membranes (Musazzi et al., 2010), suggesting an increase of the RRP of vesicles. We subjected rats to FS-stress as above and found that: (1) Serum levels of CORT were rapidly elevated in serum after acute stress (Fig. 2A); (2) Glutamate release evoked by *in vitro* depolarization of PFC/FC synaptosomes with 15 mM KCl, which promotes Ca^{2+}-dependent neurotransmitter release (Raiteri et al., 2002), was significantly enhanced in stressed rats (Fig. 2B). To assess whether acute stress increased the RRP size, we measured glutamate release evoked by hypertonic sucrose, a standard method for measuring the RRP (Rosenmund and Stevens, 1996). We found that acute stress induced a remarkable enhancement (+102.2% ± 18.44) of glutamate release evoked by hypertonic sucrose (Fig. 2C), strongly suggesting

an increase in the RRP size. No changes were observed after stress in GABA, aspartate and glycine release (data not shown), suggesting that acute stress selectively affected glutamate transmission.

To investigate whether the stress hormone CORT was able to replicate the effects of acute stress on glutamate release and to verify whether local, non-genomic mechanisms are involved in the action of CORT, we incubated *in vitro* PFC/FC synaptosomes from control rats with CORT. In these experiments, synaptosomes were preincubated with 0.02 μM [3H]D-Asp, a non-metabolizable analogue of glutamate used to label the synaptosomal glutamate releasing pools (Milanese et al., 2011). First, we measured the effect of CORT on [3H]D-Asp release evoked by *in vitro* depolarization with 15 mM KCl. For this purpose, we incubated synaptosomes for 90 seconds with 15 mM KCl in the presence of 100 nM or 10 μM CORT. Different from the effect of acute stress (Fig. 2b), and in line with the results of intracellular recordings (Fig. 1A), we found that the local application of CORT to PFC/FC synaptosomes failed to induce any change in depolarization-evoked [3H]D-Asp release (Fig. 2D). Second, we assessed whether *in vitro* application of CORT was able to affect the RRP size, measuring the effect of CORT on hypertonic sucrose-evoked [3H]D-Asp release. Synaptosomes from control rats were

incubated for 15 seconds with 250 mM sucrose in the presence of 100 nM or 10 μM CORT (Fig. 2E). We found that both concentrations of CORT markedly increased hypertonic sucrose-evoked release of [3H]D-Asp (50.000% ± 10.345 and 139.080% ± 21.839 for 100 nM and 10 μM CORT respectively). Similar results were obtained with a 500 mM sucrose pulse: 39.860% ± 9.790 and 78.322% ± 34.499 increase with 100 nM and 10 μM CORT, respectively (not shown).

These data suggest that CORT increases the RRP of vesicles with a fast mechanism, probably by increasing the number of docked vesicles. However, as suggested by the lack of increase in EPSP amplitude and PPR (Fig. 1), and in depolarization-evoked [3H]D-Asp release (Fig. 2D), these added vesicles do not seem to be fully primed for fusion and less susceptible to stimulation.

The increase of readily releasable pool of glutamate induced by corticosterone is dependent on the activation of mineralocorticoid and glucocorticoid receptors in synaptic terminals

Numerous studies have shown that the rapid effects of stress on glutamatergic transmission are mediated by non-genomic action of the stress hormone CORT, through the activation of non-cytosolic

MR and GR, putatively associated with plasma membrane (Karst et al., 2005; 2010; Groeneweg et al., 2012;Prager and Johnson, 2009).

First, we characterized the presence of both MR and GR at different cellular levels in PFC/FC, performing a Western blotting analysis on total tissue homogenate (H), nuclear fraction (P1), cytosolic fraction (S2), synaptosomes (syn), presynaptic membranes (LP1) and synaptic spyne membranes (triton-insoluble fraction, TIF). We confirmed the expression of both receptors in H, P1 and in S2, consistent with their classical cytosolic localization, but also demonstrated the localization of the two receptors at synaptic level, both at presynaptic (LP1) and postsynaptic membranes (TIF) (Fig. 3A).

Then, to evaluate if the increase of RRP size induced by CORT in synaptosomes was due to a local effect of CORT on MR or GR, control synaptosomes were incubated with a selective antagonist of the two receptors (SPIR or RU486, respectively) (Karst et al., 2005; 2010) during stimulation with 100 nM CORT and sucrose pulse. We found that both SPIR and RU486 were able to completely prevent the increase of RRP induced by CORT (Fig. 3B) and that the effects of the two antagonists were not additive. These data suggested that

the rapid effects of CORT on the RRP size are mediated by GR and MR located on synaptic terminals.

In vitro application of corticosterone increases the number of FM1-43 fluorescent synaptosomes in the TIRFM field through the activation of mineralocorticoid and glucocorticoid receptors

To further evaluate whether the increase in the RRP size (see Fig. 2E) was due to a direct effect of CORT on SV mobilization in PFC/FC synaptic terminals, we used TIRFM, a technique that allows the study of events occurring in a small interval (about 100 nm) below the plasma membrane (Groves et al., 2008).

We purified synaptosomes from PFC/FC of control rats, and labeled SV with the lipophilic dye FM1-43 in the presence of 25 mM KCl (see Materials and Methods for details, Serulle et al., 2007). FM1-43 selectively labels the recycling pool of vesicles and has been widely used to monitor the kinetics of SV release and recycling. PFC and FC are brain areas where glutamate neurons and synapses are predominant (Sanacora et al., 2012; Douglas and Martin, 2007). To confirm that the majority of isolated synaptosomes in our preparation were glutamatergic, we measured the amount of glutamate positive spots with respect to the total FM1-43 labeled structures, co-labeling

synaptosomes with both FM1-43 FX (fixable analog of FM1-43) and a glutamate antibody. Glutamate-stained FM1-43 FX spots were 62.76%, ± 1.76 SEM of total; Fig. 4A), indicating that the majority of FM1-43-labeled vesicles are glutamatergic.

When visualized by TIRFM, FM1-43 staining defines fluorescent spots, which correspond to synaptosomes containing fluorescent SV located next to the plasma membrane (Fig. 4A,B). Therefore, we incubated FM1-43 stained synaptosomes with different concentrations of CORT (100 nM, 1 µM, 10 µM) or with DMSO and recorded them under TIRF illumination for 10 min. We found that CORT caused an increase in the number of fluorescent spots in the TIRFM field, suggesting an accumulation of SV in close proximity to the synaptic membranes (Fig. 4B; Movies 1 and 2 in Suppl. Results). The increase in the number of fluorescent spots was significant for all CORT concentrations, and started immediately after addition of CORT, as shown in Fig. 4C. The areas under the curve (AUC) values were maximal (+29.854% ± 6.160 for 100 nM CORT, +30.893% ± 8.486 for 1 µM CORT, +36.015% ± 11.647 for 10 µM CORT, Fig. 4D) after 5 min and remained constant until the end of recording after 10 min (+32.255% ± 6.083 for 100 nM CORT, +32.129% ± 8.941 for 1 µM CORT, +39.311% ± 11.541 for 10 µM CORT, Fig. 4E).

These results suggest that *in vitro* incubation of PFC/FC synaptosomes with CORT induces a rapid mobilization of SV towards the presynaptic membrane, a result in line with the increase in RRP size shown above (see Fig. 2E).

In order to assess if the vesicle mobilization mediated by CORT was downstream of MR or GR activation, we performed the TIRFM experiments in the presence of selective receptor antagonists. We first assessed whether SPIR or RU486, in the absence of CORT, affected SV mobilization. As shown in Suppl. Fig. 1A,B, the number of labeled spots visualized in the TIRFM field was not changed by the two compounds. Therefore, synaptosomes were pre-incubated for 5 min with MR/GR antagonists and then 10 μM CORT was added for 10 min. The presence of SPIR and RU486 before and during incubation with CORT was able to completely abolish the effect of CORT. Both antagonists blocked the increase in the number of fluorescent spots induced by CORT (-32.44% ± 11.833 for SPIR, and -20.66% ± 5.587 for RU486, Fig. 4F-H). These data suggest the involvement of both MR and GR in the SV mobilization induced by CORT.

Both acute stress and corticosterone increase the phosphorylation levels of Synapsin I at site 1 in synaptic membranes of PFC/FC synaptosomes

We evaluated a possible role of synapsin I (an abundant presynaptic protein involved in SV mobilization and in the modulation of exocytotic events) in the presynaptic effects of CORT. Synapsin I was chosen because its function is regulated by seven different consensus sites for phosphorylation activated by different protein kinases (Greengard et al., 1993); the identification of select phosphorylation changes in synapsin I may give clues as to the presynaptic signaling pathways affected by CORT.

Synaptosomes from control rats were incubated with either 10 μM CORT or DMSO for 10 minutes, lysed and subjected to Western analysis. First, we measured phosphorylation levels of synapsin I in total synaptosomes. We did not find any changes in both total expression level and phosphorylation levels at sites 1 and 3 to 7 (currently there is no antibody available for site 2) (Fig. 5A). Since synapsin I also plays crucial roles, in the control of SV docking, fusion and recycling at active zones (Cesca et al., 2010), we purified presynaptic membranes, which contain the active zone and the RRP of SV, from synaptosomes incubated with CORT. We found that,

although synapsin I expression level was still unchanged, the incubation of synaptosomes with CORT induced a significant (+26.2% ± 8.752) increase of phosphorylation of synapsin I in presynaptic membranes, selectively at site 1 (Ser9; Figure 5B), suggesting a membrane-located role of this protein in the presynaptic action of CORT. As in total synaptosomes, the phosphorylation levels at other sites (3-7) of the protein were unchanged also in presynaptic membranes (Figure 5B).

To assess if this modulation of synapsin I phosphorylation induced by CORT *in vitro* was also observed in acutely stressed animals, we purified synaptosomes from PFC/FC of control and FS-stressed animals and measured expression and phosphorylation levels of synapsin I, as above. We did not find any changes both in synapsin I expression levels and phosphorylation levels at site 1, and 3 to 7 in synaptosomes (Figure 6A), as reported in experiments with CORT. However, when the Western blot analysis was conducted on presynaptic membranes, we found a similar increase (+25.28% ± 7.650) of phosphorylation levels of synapsin I selectively at site 1, with no changes in total expression and phosphorylation at other sites, as previously shown after *in vitro* incubation with CORT (Figure 6B).

Activation of site 1 phosphorylation of synapsin I in synaptic membranes by CORT requires mineralocorticoid and glucocortcoid receptors

To assess if the increase in the phosphorylation level at site 1 of synapsin I induced by CORT was due to the activation of synaptic MR and/or GR, synaptosomes were pre-treated for 5 minutes with SPIR or RU486 and then incubated with CORT for 10 minutes in the continuous presence of the antagonists. As shown in Fig. 7, both SPIR and RU486 significantly reduced the increase of synapsin I phosphorylation in synaptic membranes (~-20%). These experiments suggest that the phosphorylation at site 1 of synapsin I is downstream of MR and GR activation in synaptic terminals

DISCUSSION

Convergent lines of evidence from rodent studies have shown that different stressors acutely increase the efflux of glutamate in cortical and limbic brain areas, including hippocampus, amygdala and PFC, and that this effect is at least partly mediated by CORT (Bagley and Moghaddam, 1997; Lowy et al. 1993, 1995; Moghaddam, 1993; Musazzi et al., 2010; Reznikov et al., 2007; Sanacora et al., 2012; Venero and Borrell, 1999). In addition, it has been shown that application of CORT *in vitro* to hippocampal slices rapidly enhances the frequency of mini-EPSPs and reduces PPF in CA1 pyramidal neurons. In these cells the hormone boosts glutamate release probability, a non-genomic effect mediated by MR located at or near the plasma membrane (Karst et al., 2005; Joels et al., 2012). However, although different stress protocols acutely trigger glutamate release in PFC (see above), it is not known whether CORT is able to replicate this action of stress in cortical areas. We asked this question in the present work, by recording in layer III pyramidal neurons of mPFC EPSPs evoked by extracellular stimulation of layer V (the same synapses where we previously found enhancement of glutamate release by acute stress; Musazzi et al., 2010), after application of CORT *in vitro*. The present results showed that neither

basal synaptic transmission nor PPF were altered by CORT (Fig. 1) clearly showing that, contrary to hippocampus, CORT by itself does not enhance glutamate release in PFC.

Our previous results showing that acute stress increases the accumulation of SNARE complex in presynaptic membranes suggested that CORT-dependent action of stress on glutamate release could be mediated by an increase of the RRP of glutamate vesicles (Musazzi et al., 2010; Popoli et al., 2012). Therefore, by using purified synaptosomes in superfusion, we measured changes in both depolarization-evoked glutamate release and the size of the RRP, after acute stress in rats or application of CORT *in vitro* to control synaptosomes. We found that while stress increased both, CORT was only able to increase the RRP, and this effect was dependent on MR/GR receptors located on purified synaptic terminals (Fig. 2-3). By using FM1-43-labeled synaptosomes visualized with TIRF microscopy, we found that application of CORT *in vitro* in a matter of minutes mobilized a quota of SV to the vicinity of the presynaptic membrane (Fig. 4), confirming that CORT rapidly increases the number of vesicles on or near the presynaptic membrane and that this is dependent on local MR/GR. Finally, we found that both acute stress and application of CORT in vitro

increased in presynaptic membranes the selective phosphorylation of site 1 (Ser9) of synapsin I, an abundant presynaptic protein involved in SV mobilization and in the modulation of exocytotic events (Fig. 5,6). This selective phosphorylation was also dependent on the activation of MR/GR.

Overall, while suggesting that the effect of acute stress on glutamate release in PFC/FC is mediated by the action of CORT on MR/GR located at presynaptic terminals, our combined results also show that CORT does not fully replicate the effect of stress on glutamate release in these cortical areas. CORT rapidly increases the number of glutamate vesicles available for release, an event that seems to be necessary for the enhancement of release induced by stress, but does not enhance the release by itself. This postulates that additional effectors in PFC/FC, in combination with CORT, mediate the complete effects of stress that result in the enhancement of glutamate release. This is at variance from hippocampus, where the local application of CORT is sufficient to induce a rapid enhancement of glutamate release and of synaptic transmission (Karst et al., 2005; Joels et al., 2012), and suggests that the mechanism whereby acute stress potentiates glutamate synaptic transmission is different in PFC/FC and hippocampus. We found that different concentrations of

CORT exerted a quantitatively similar effect on the mobilization of SV as recorded by TIRF microscopy (see Fig. 4C). These data, in one with the results showing that both the selective antagonists of MR and GR block the build-up of RRP, the mobilization of SV and the phosphorylation of site 1 of synapsin I, suggest that these processes involve multiple receptors with different affinities for CORT. We observed for the first time that two receptors visualized by antibodies for classical MR and GR are present in purified synaptosomes, as well as in presynaptic membranes (LP1) and postsynaptic spine membranes (TIF). The presence of similar receptors at pre-and postsynaptic structures has been previously demonstrated in amygdala (Prager et al., 2010). Putative membrane-associated MR/GR have been described by several authors, although the mechanism of their association with membranes and their downstream signaling awaits characterization (for a discussion, see Joels et al., 2012).

Ser9 of synapsin I is a consensus site for phosphorylation by cAMP-dependent protein kinase (PKA) or calcium/calmodulin-dependent protein kinase I (CaMKI) (Cesca et al., 2010). Future experiments are warranted to understand which kinase pathway is responsible for CORT-induced phosphorylation of synapsin I and how this signaling

is linked to MR or GR. The observation that both acute stress and application of CORT *in vitro* to synaptosomes elicit the selective phosphorylation of the same site 1 among the seven different phosphorylation sites of synapsin I strongly suggests that this molecular change is involved in the action of stress on the presynaptic machinery. Synapsin I is a multi-regulated protein and its function in the presynaptic machinery, although studied for several years, is still controversial and open to discussion. Synapsin I has been involved in the regulation of SV mobility, of release probability during high-frequency stimulation, in stabilization of SV structure, and in regulation of endocytosis (Greengard et al., 1993; Sun et al., 2006; Rosahl et al., 1995; Bloom et al., 2003). It is now believed that a portion of the protein does not dissociate from SV, and remains associated with the RRP, where synapsins might contribute to one or more steps in the docking, post-docking and fusion events, regulating the size of the RRP and facilitating the priming and/or fusion steps (Cesca et al., 2010). It will be interesting to study how synapsin I is involved in the effect of stress.

In summary, although the enhancement of glutamate release induced by acute stress is mediated by CORT (Musazzi et al., 2010;

Popoli et al., 2012), the hormone is necessary but not sufficient for this effect in PFC/FC. CORT rapidly induces mobilization of SV, which builds up the RRP and creates the premises for enhancement of glutamate release. However, additional effectors, such as a retrograde messenger (Hill and McEwen, 2009), may be necessary to carry out the complete effect of stress in these cortical areas. Therefore, although acute stress enhances glutamate release and synaptic transmission in PFC/FC as in hippocampus, the mechanisms involved seem to be different. The dissection of the mechanism whereby stress enhances glutamate release in PFC/FC may allow to understand better the role of these brain areas in the stress response. Moreover, recent developments have identified in the different sites of regulation of the glutamate synapse potential targets for intervention, which could allow more efficacious and faster treatment of neuropsychiatric disorders (Sanacora et al., 2012; Popoli et al., 2012).

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FIGURE LEGENDS

Figure 1 (A) (Top graph) Application of 10 uM CORT does not alter basal synaptic transmission. The results are shown as mean values plus standard error for a group of n = 6 recordings in the same conditions. (Bottom graph) the resting potential of the neuron was measured as mean value of the membrane potential in the 50ms preceding the stimulus and then averaged in groups of six (one sample for each minute of recording). A group of three synaptic responses is shown on the top of the figure, before (left) and after (right) the application of 10uM CORT. (B) Application of 10 uM CORT does not alter paired-pulse facilitation of synaptic transmission. The analysis results are shown as mean values plus standard error for a group of n = 2 recordings in the same conditions: the paired-pulse ratio was computed as the amplitude ratio between the first and the second EPSPs (measured as peak-to-baseline difference) and then averaged in groups of six (one sample for each minute of recording). A group of three paired-pulse synaptic responses is shown on the top of the figure, before (left) and after (right) the application of 10uM CORT.

Figure 2 (A) CORT serum levels in vehicle-treated (control, CNT) and subjected to FS-stress (STRESS) rats. Data are expressed as means±SEM. *** $p<0.0001$ vs CNT, Student's t test for unpaired samples (n = 8 rats/group). (B) 15 mM KCl-evoked endogenous glutamate release from superfused PFC/FC synaptosomes of vehicle-treated and subjected to acute FS-stress rats. (C) 250 mM sucrose-evoked endogenous glutamate release from superfused PFC/FC synaptosomes of vehicle-treated (control, CNT) and subjected to acute FS-stress rats. Data are expressed as mean ± SEM. Student's t test for unpaired samples * $p<0.05$,*** $p<0.001$ n = 7/8.

(D) 15 mM KCl-evoked [3H]D-Asp release from superfused PFC/FC synaptosomes of control rats incubated with 0.01% DMSO or 100 nM, 10 μM CORT, during the KCl pulse (90 seconds), (E) 250 mM sucrose evoked [3H]D-Asp from superfused PFC/FC synaptosomes of control rats incubated with 0.01% DMSO or 100 nM, 10 μM CORT, during the hypertonic sucrose pulse (15 seconds). Data are expressed as mean ± SEM. One-Way ANOVA followed by Neuman-Keuls post-hoc test.** $p<0.01$,*** $p<0.001$ n = 4/11.

Figure 3 (A) Immunoreactive bands obtained by Western blotting of PFC/FC MR and GR in 10 µg total homogenate (H), 10 µg nuclei-associated membrane (P1), 20 µg cytosolic (S2), 20 µg synaptosomes (syn), 20 µg presynaptic membranes (LP1) and 20 µg synaptic spine membranes (triton insoluble fraction, TIF). (B) 250 mM sucrose evoked [3H]D-Asp release from superfused PFC/FC synaptosomes of control rats incubated with 0.01% DMSO or 100 nM CORT, and incubated with 100 nM SPIR or 500 nM RU486 during CORT incubation. Data are expressed as mean ± SEM. One-Way ANOVA followed by Neuman-Keuls post-hoc test. * $p<0.01$ CORT vs DMSO; § $p<0.001$ RU486, RU486+SPIR vs CORT, # $p<0.05$ SPIR vs CORT n = 4/11.

Figure 4 (A) i-ii Evaluation of the percentage of glutamatergic synaptosomes in TIRFM experiments. iii Graph showing the percentage of glutamate positive (black column) or glutamate negative (white column) over FM1-43 positive spots visualized in TIRFM. (B) Effect of CORT on the number of FM1-43 fluorescent spots visualized by TIRFM. Representative TIRFM images (magnification 100 X): synaptosomes incubated with 0.01% DMSO or 10 µM CORT. Only the synaptosomes incubated with CORT show

an increase in the fluorescent spots appearing in TIRFM field after 5 min and 10 minutes, compared with the number of spots at t = 0 minute. (C) Graph showing the number of spots visualized in TIRFM (expressed as percentage vs the number of spots at t = 0 minute) during 10 min of *in vitro* incubation with DMSO (0.01%) or CORT (10 µM; 1 µM; 100 nM). Data are expressed as mean ± SEM n = 6/11 recordings from 4 independent experiments. (D-E) Histograms representing the AUC of the recording curves (Fig. 4C) from t = 0 minute to t = 5 minutes (D) and from t = 0 minute to t = 10 minutes (E). AUC are expressed as percentage vs the AUC value of DMSO condition. Data are expressed as mean ± SEM. One-Way ANOVA followed by Neuman-Keuls post-hoc test showed a significant effect of 10 µM, 1 µM, 100 nM CORT vs DMSO ($p<0.01$); n= 6/11 recordings from 4 independent experiments.

(F, G, H) Effect of SPIR and RU486 on CORT induced increase of FM1-43 fluorescent spots visualized by TIRFM. (F) Graph representing the number of spots visualized in TIRFM (expressed as percentage vs the number of spots at t = 0 minute) during 10 minutes of *in vitro* incubation with 0.1% DMSO or 10 µM CORT after pre-incubation with SPIR or RU486. Data are expressed as mean ± SEM; n = 5/7 recordings from 3 independent experiments. (G-H)

Histograms representing the AUC of the recording curves (Fig. 4F) from t = 0 minute to t = 5 minutes (G) and from t = 0 minute to t = 10 minutes (H). AUC are expressed as percentage vs the AUC value of DMSO condition. Data are expressed as mean ± SEM. One-Way ANOVA followed by Neuman-Keuls post-hoc test showed a significant effect of CORT vs DMSO ($p<0.05$) and SPIR and RU486 vs CORT ($p<0.05$); n = 4/7 recordings from 3 independent experiments.

Figure 5 Expression and phosphorylation levels at site 1 of synapsin I in PFC/FC whole synaptosomes and synaptic membranes from synaptosomes of control rats incubated with DMSO or 10 μM CORT for 10 minutes. (A) Western blotting analysis of total synapsin I and phosphorylation levels of phospho-Ser^{9} (site 1), phospho-Ser^{603} (site 3), phospho-Ser^{62-67} (site 4, 5), phospho-Ser^{549} (site 6), phospho-Ser^{553} (site 7) of synapsin I in PFC/FC synaptosome. (B) Western blotting analysis of total synapsin I and phosphorylation levels of phospho-Ser^{9} (site 1), phospho-Ser^{603} (site 3), phospho-Ser^{62-67} (site 4, 5), phospho-Ser^{549} (site 6), phospho-Ser^{553} (site 7) of synapsin I in synaptic membranes.

Western blotting band intensities were normalized for β-actin. Data are expressed as percentage immunoreactivity vs DMSO (mean ± SEM). Student's t test for unpaired samples n = 6 in duplicate or triplicate. For phosphoylation level of phospho-Ser9 (site 1) of synapsin I in synaptic membranes Student's t test for unpaired showed a significant effect of CORT vs DMSO (** $p<0.01$ CORT vs DMSO).

Insets: representative immunoreactive bands from Western blots.

Figure 6 Expression and phosphorylation levels of synapsin I in PFC/FC synaptosomes and synaptic membranes from PFC/FC of vehicle-treated (control, CNT) and subjected to FS-stress (STRESS) rats. Western blotting analysis of total synapsin I expression and phosphorylation levels of phospho-Ser^{9} (site 1), phospho-Ser^{603} (site 3), phospho-$Ser^{62\text{-}67}$ (site 4, 5), phospho-Ser^{549} (site 6), phospho-Ser^{553} (site 7) of synapsin I in PFC/FC synaptosomes. (B) Western blotting analysis of total synapsin I expression and phosphorylation levels of phospho-Ser^{9} (site 1), phospho-Ser^{603} (site 3), phospho-$Ser^{62\text{-}67}$ (site 4, 5), phospho-Ser^{549} (site 6), phospho-Ser^{553} (site 7) of synapsin I in synaptic membranes.

Western blotting band intensities were normalized for β-actin. Data are expressed as percentage immunoreactivity vs CNT (mean ± SEM). Student's t test for unpaired samples n = 12 in duplicate or triplicate. For phosphorylation level of phospho-Ser9 (site 1) of synapsin I in synaptic membranes Student's t test for unpaired showed a significant effect of STRESS vs CNT (** $p<0.01$ STRESS vs CNT).

Insets: representative immunoreactive bands from western blotting experiments.

Figure 7 Effects of pre-treatment with SPIR or RU486 on phosphorylation levels of synapsin I (site 1) in PFC/FC synaptic membranes from synaptosomes incubated with DMSO or CORT.

(A) Western blot analysis of phospho-Ser^9 (site 1) synapsin I in synaptic membranes from synaptosomes incubated with DMSO, CORT or pre-treated with SPIR or RU486 and incubated with CORT. The band intensities were normalized for β-actin. Data are expressed as percentage immunoreactivity vs DMSO (mean±SEM). Data are expressed as mean±SEM. One-Way ANOVA followed by Neuman-Keuls post-hoc test. * $p<0.05$,** $p<0.01$ n = 8/18 in duplicate or quadruplicate.

Supplementary Figure 1 Effect of spironolactone (SPIR) and RU486 on FM1-43 fluorescent spots visualized by TIRFM. (A) Graph representing the number of spots visualized in TIRFM (expressed as percentage vs the number of spots at t = 0 minute) during 5 minutes of *in vitro* incubation with SPIR or RU486. Data are expressed as mean ± SEM; n = 4/7 recordings from 3 independent experiments. (B) Histogram representing the AUC of the recording curves (Suppl Fig. 1) from t = 0 minute to t = 5 minutes (AUC are expressed as percentage vs the AUC value of DMSO condition). Data are expressed as mean ± SEM. One-Way ANOVA followed by Neuman-Keuls post-hoc test did not show any effects of both SPIR and RU486; n = 4/7 recordings from 3 independent experiments

Figure 1

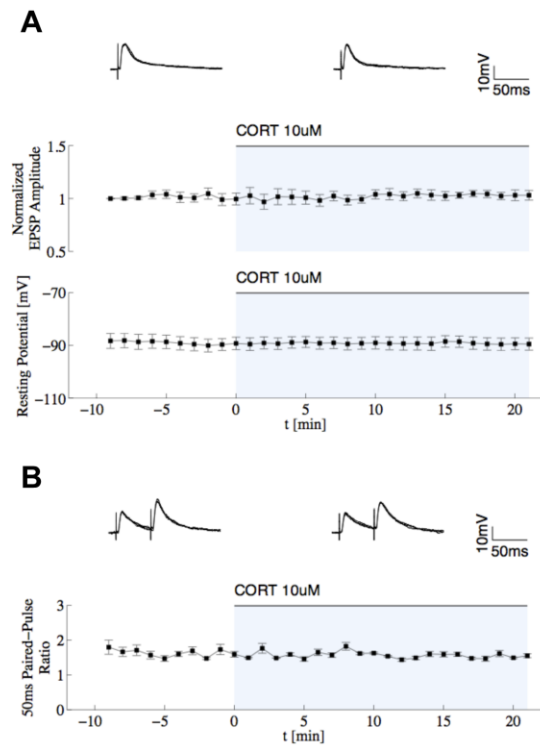


Figure 2

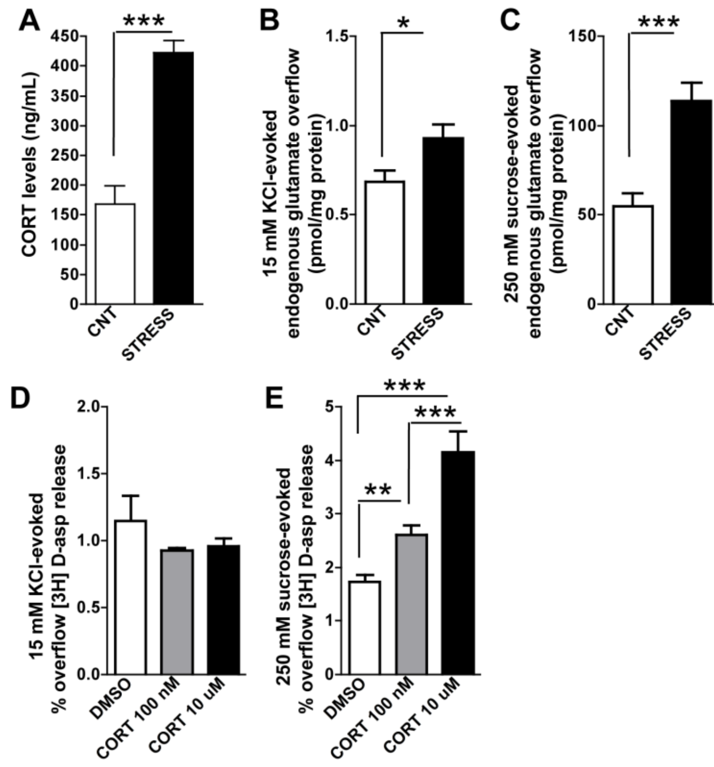


Figure 3

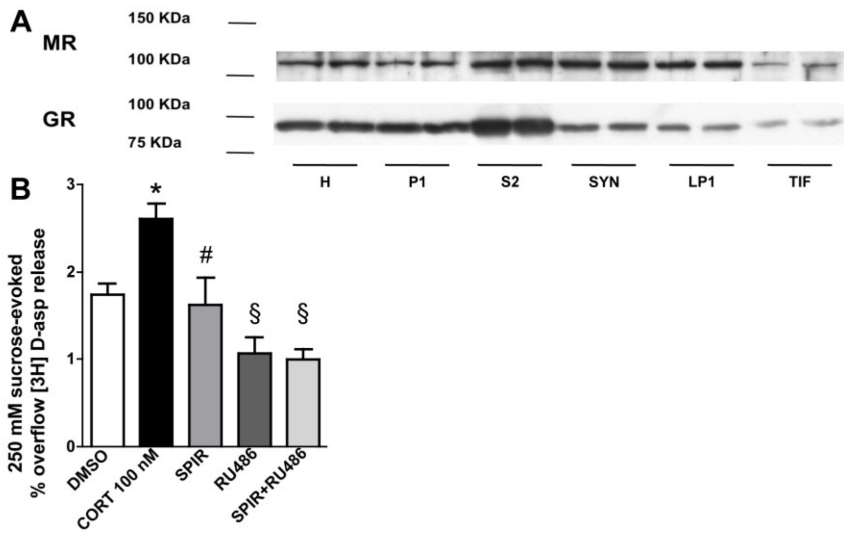


Figure 4

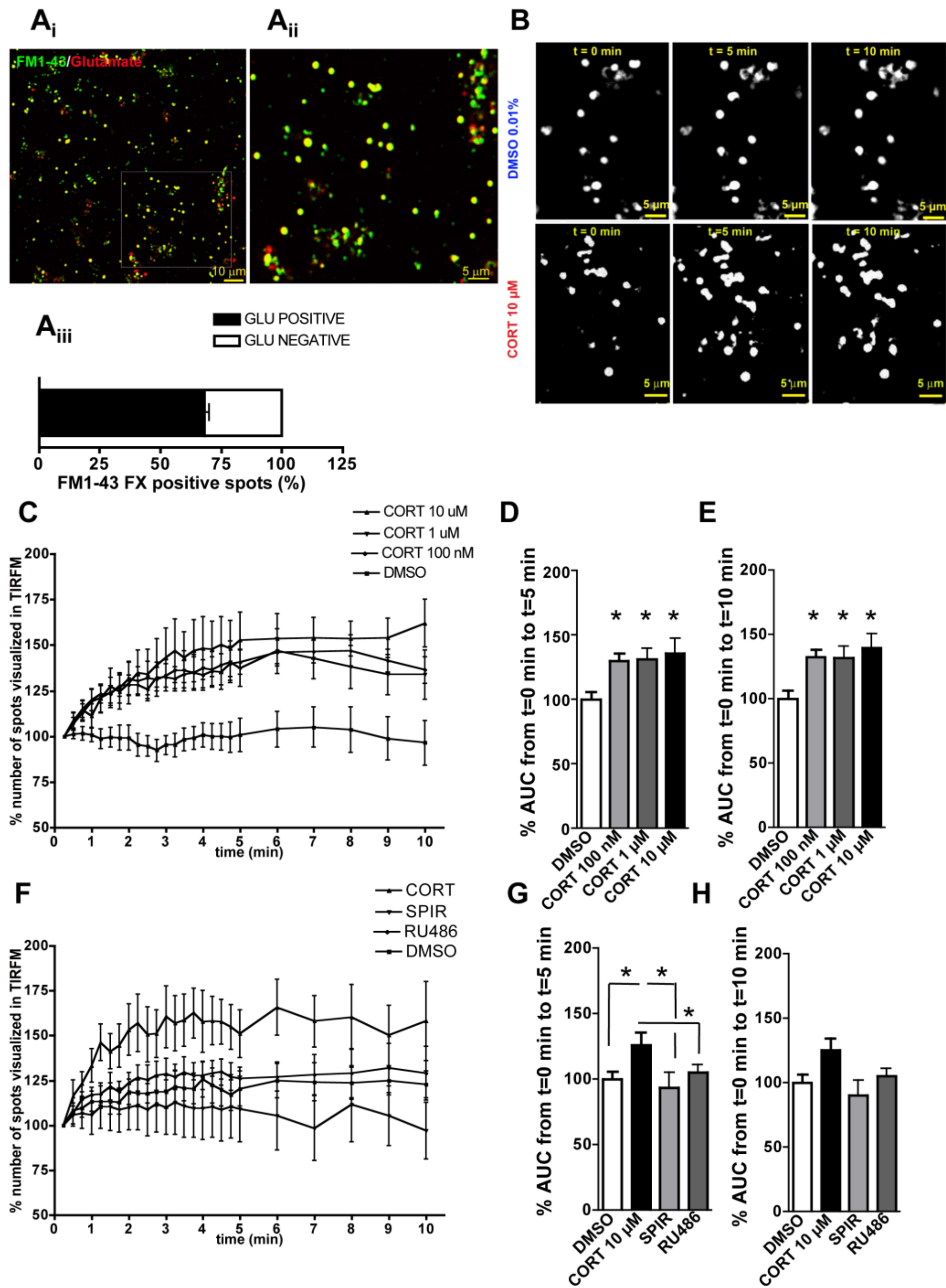
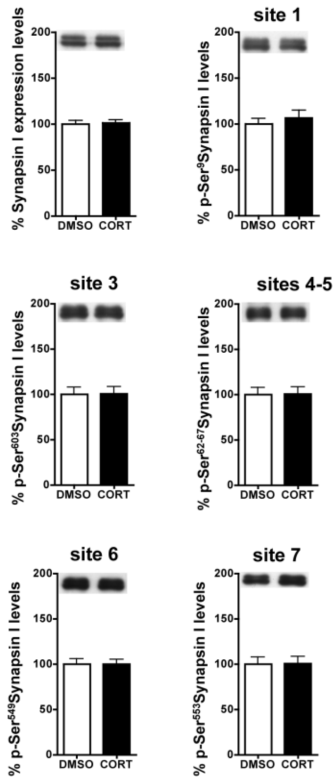


Figure 5

A



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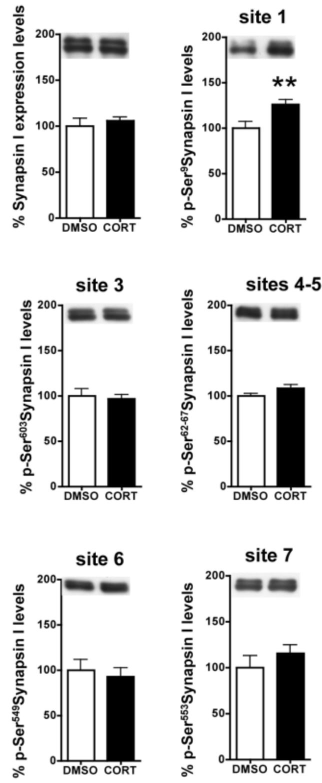


synaptosomes

synaptic membranes

Figure 6

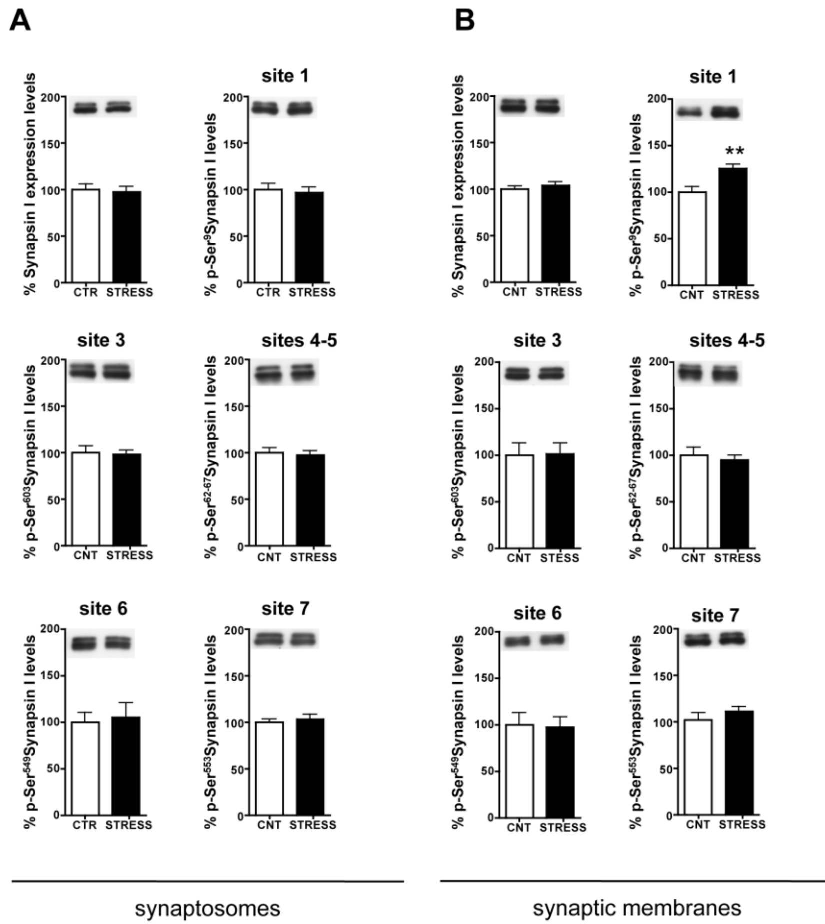
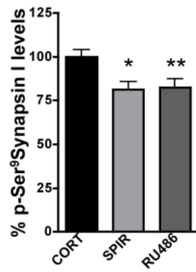
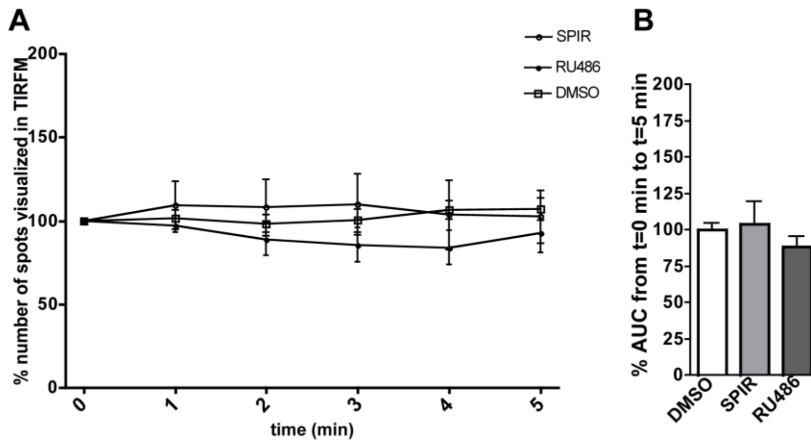


Figure 7



Supplementary Figure 1



GENERAL DISCUSSION AND CONCLUSIONS

Numerous studies have clearly underlined the importance of glutamatergic transmission in mood disorders. The dysregulation of glutamatergic transmission is now considered a core feature of the pathology and an interesting target for novel therapeutic approaches (Sanacora et al., 2008). Recent studies have demonstrated that drugs that directly target different sites of regulation of the glutamate synapse may have antidepressant effects. In particular, preclinical and clinical studies have focused on antidepressant effects of antagonists of NMDA receptors, positive modulators of AMPA receptors and mGluR agonists/antagonists. In this line of research, an exciting example was the observation that sub-anesthetic doses of intravenously infused ketamine (a non competitive NMDA antagonist) produced a rapid and sustained antidepressant effect on individuals with treatment-resistant depression (Zarate et al., 2006). The fast effects of ketamine have inspired new lines of preclinical research to explore the underlying neuronal circuitries and downstream signaling connected with glutamatergic transmission. In addition to drugs that directly affect glutamate receptors, it is now clear that all major sites of regulation of the glutamate synapse (including presynaptic release machinery, postsynaptic ionotropic receptors, pre-postsynaptic metabotropic receptors, glutamate reuptake and glutamate/glutamine recycling mechanisms) represent, at the same time, targets of traditional ADs with different primary mechanisms. A further example is the finding that the presynaptic machinery of glutamate release is regulated by traditional ADs in the acute stress response (Musazzi et al., 2010). Indeed, these drugs block the stress-induced increase of glutamate release, without

blocking the rise of CORT levels and glutamatergic RRP size (Musazzi et al, 2010; Popoli et al., 2012). It has been proposed that ADs can attenuate the exaggerated or inadequate stress response (Musazzi et al., 2011). The effects of stress and glucocorticoids in the brain and in particularly on glutamate transmission are highly complex and far to be completely understood. It is now known that acute stress in rodents rapidly enhances glutamate release/transmission in selected brain areas, like hippocampus, amygdala and PFC, but repeated stress, particularly in PFC, seems to have the opposite effect, inducing dendritic retraction, loss of spines/synapses and decrease of glutamatergic transmission (Popoli et al. 2012). However, the mechanism by which stress, and in particular the major stress hormone CORT, modulate presynaptic glutamate release in PFC/FC is still not clear.

We have demonstrated that in synaptosomes from PFC/FC, both stress and CORT increase sucrose-evoked glutamate release, suggesting an increase of RRP of vesicles. Interestingly, we found that only acute stress induced an increase in depolarization-evoked glutamate release, whereas CORT did not alter glutamate release evoked by depolarization in synaptosomes and did not affect EPSPs in slices of mPFC. These findings confirm that CORT is a key component of the stress response, necessary for the increase of RRP, but not sufficient to trigger release of glutamate (see below). We have also demonstrated that the effect of CORT on RRP is dependent on MR and GR located on purified synaptic terminals, indeed both selective GR and MR antagonists blocked the increase of sucrose-evoked glutamate release. Moreover, by using FM1-43-

labeled synaptosomes visualized with TIRF microscopy, we have shown that application *in vitro* of CORT induced an increase of vesicle mobilization towards the presynaptic membrane, that is consistent with enhancement of the RRP. This effect was also dependent on the activation of MR and GR. At molecular level, we have found that CORT, through the activation of MR and GR, induced an increase in the selective phosphorylation at site 1 of synapsin I in synaptic membranes. Moreover, we have found that also acute stress increased the phosphorylation of the same site 1 of synapsin I, suggesting that this molecular change is involved in the action of stress on the presynaptic machinery. The role of synapsin I has been studied for a long time, it is known that the protein clusters synaptic vesicles in a reserve pool, away from the plasma membrane, by acting as a linker between synaptic vesicles and actin cytoskeleton. Upon stimulation, synapsin I is phosphorylated and dissociates from the reserve pool, allowing vesicles to move close to the active zone, containing the RRP (Cesca et al., 2010). However, it is now believed that a quota of synapsin protein does not dissociate from synaptic vesicles and is still present in the RRP, where it probably contributes to docking, post-docking and fusion events (Cesca et al., 2010).

The rapidity of these effects (minutes) and the absence of nuclear DNA in synaptosomes clearly suggest that the fast effects of CORT are mediated by synaptic (non-genomic) mechanisms probably through membrane-associated MR and GR. We showed that MR and GR are expressed at both pre- and postsynaptic level in PFC/FC; the expression at pre- and postsynaptic level of the two receptors has

been previously reported also in amygdala (Prager et al., 2010). These findings are in line with the rising literature regarding the fast non-genomic effects of CORT in different brain areas, which may be responsible for the first phase of the fight-or-flight response, without affecting in the first several minutes, delayed genomic mechanisms (Jöels et al., 2012).

In conclusion, differently from hippocampus, where CORT was shown to increase glutamatergic transmission via non-genomic MR-mediated mechanisms (Karst et al., 2005; Jöels et al., 2012), we have demonstrated that in PFC/FC CORT only promotes an increase of the RRP size, without triggering release, suggesting that other neurotransmitters or mediators released during the stress response are necessary to enhance glutamate transmission (Figure 5). This means that the synaptic, non-genomic effects of CORT, changing the number of vesicles ready for release, represent a first key step in the plastic modulation of the glutamatergic synapse induced by acute stress. However, this necessary role of CORT needs the activation of further mechanisms able to increase the probability of vesicle release to induce an increase of presynaptic glutamate release.

If this stress-induced increase of glutamate release is inadequate and not stabilized, a process leading toward maladaptative changes in structure and function of the glutamate system may occur.

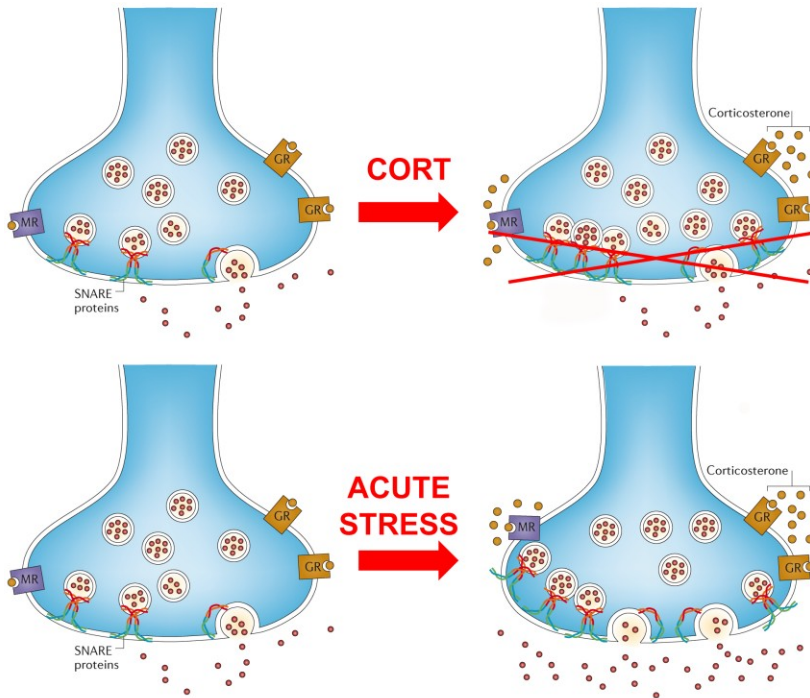


Figure 5. Effect of CORT and acute stress on glutamate synapse
The figure illustrates the different effects of CORT and acute stress on glutamate synapse. CORT increases the size of RRP of vesicles, but does not trigger release, whereas acute stress promotes both the increase of RRP and depolarization-dependent glutamate release (see text for details).

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