ROLE OF GLUTAMATE TRANSPORTERS AND GLUTAMATERGIC SYSTEM IN CORTICAL AND LIMBIC BRAIN AREAS IN AN ANIMAL MODEL OF DEPRESSION

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Abbreviations

3ChT	Three-chambered social interaction test
5-HT	Serotonin
ACTH	Adrenocorticotropin
AM	Morning
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Amy	Amygdala
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BW	Body weight
CaMKIIα	Calcium/calmodulin-dependent protein kinase type II subunit alpha
CNS	Central nervous system
COMT	Cathecol-O-methyltransferase
Con	Control
CRH	Corticotropin-releasing hormone
CSDS	Chronic social defeat stress
DA	Dopamine
DAT	Dopamine transporter
DI	Discrimination index
DNA	Deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5th edition
EAAT	Excitatory amino acid transporter
GABA	*gamma*-Aminobutyric acid
GC	Glucocorticoid
GRs	Glucocorticoid receptors
$G_{i/o}$	G_i protein alpha subunit
GPCR	G-protein-coupled receptor
G_q	G_q protein alpha subunit

GWAS	Genome-wide association studies
h	hour
HPA	Hypothalamo-pituitary-adrenocortical
Hpc	Hippocampus
KO	Knockout
LTD	Long-term depression
LTP	Long-term potentiation
MAO	Monoamine oxidase
max_{val}	Maximum value
MDD	Major depressive disorder
Mg^{2+}	Magnesium
mGluR	Metabotropic glutamate receptor
min	minute
min_{val}	Minimum value
mPFC	Medial prefrontal cortex
MRs	Mineralocorticoid receptors
NAc	Nucleus accumbens
NE	Norepinephrine
NET	Norepinephrine transporter
NMDA	*N*-Methyl-d-aspartic acid
NPY	Neuropeptide Y
OFT	Open field test
OLM	Object location memory
ORM	Object recognition memory
p	Probability
PET	Positron emission tomography
PFC	Prefrontal cortex
PLC	Phospholipase C
PM	Afternoon/evening
PVN	Paraventricular nucleus
Res	Resilient
SEM	Standard error of the mean
SERT	Serotonin transporter
shNAc	Nucleus accumbens shell
SI	Social interaction
SNPs	Single-nucleotide polymorphisms

SPT	Sucrose preference test
Str	Dorsal striatum
Sus	Susceptible
SV2A	Synaptic vesicle glycoprotein 2A
t	Time
TH	Tyrosine hydroxylase
TST	Tail suspensión test
UCMS	Unpredictable chronic mild stress
V	Volume
vHpc	Ventral hippocampus
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
WT	Wild type

Abstract

Major depressive disorder affects around 5% of the world´s population. Most antidepressant drugs have focused mainly on monoamine neurotransmitters synaptic levels in the brain such as serotonin, noradrenaline and dopamine. However, the delayed latency of the therapeutic actions of antidepressants and the poor efficacy for some subpopulations of patients suggest that mechanisms beyond monoaminergic modulation at synapses may be involved in the antidepressant actions. Several lines of evidence indicate that the pathophysiology of depression is associated with dysregulation of glutamate system and clearance mechanisms in brain regions mediating cognitive-emotional behaviors. Chronic stress results in an increase in extracellular glutamate and dysregulation of the glutamatergic system in cortical and limbic brain areas in patients with MDD and animal models of depression. However, the mechanisms underlying the abnormal glutamatergic transmission in depression are incompletely understood. The excitatory amino acid transporter 3 (EAAT3) - a member of the high-affinity glutamate transporters - which plays an essential role in transporting glutamate across plasma membranes in neurons, and in maintaining extracellular glutamate concentrations below neurotoxic levels, may have a pivotal role in dysregulation of glutamatergic signaling associated to depression.

This study aims to evaluate the consequences of unpredictable chronic mild stress (UCMS) on the expression of glutamate transporters and ionotropic receptors in the cortical-limbic brain areas in wild type (WT) mice; and to determine if increased EAAT3 expression in the forebrain in mice can reduce susceptibility to UCMS. On the other hand, we evaluated the consequences of psychosocial stress at the behavioral level in WT mice subjected to chronic social defeat stress (CSDS). WT mice subjected to UCMS and control group were tested to anxiety- and depressive-like behaviors. Moreover, we evaluated long-term memory using object location and recognition tasks. Protein levels of AMPA and NMDA receptors subunits and glutamate transporters were analyzed by western blot. Mice with EAAT3 overexpression driven by CaMKIIα-promoter (EAAT3^{glo}/CMKII) and control (EAAT3^{glo}) littermates were assessed to anxiety- and depressive-like behaviors, and memory tests in baseline and UCMS conditions.

Longer immobility time was observed in the tail suspension test in WT mice susceptible to CSDS. However, no significant differences were found between resilient and susceptible individuals in anxiety-like behaviors and anhedonia. Apparently, social defeat in WT mice would only be concerning sociability to an unknown aggressive mouse and the despair behavior.

In WT mice, we observed that chronic stress induced anxiety- and depressive-like behaviors and deficits in memory tests, in addition to increased EAAT1, NMDA receptors GluN2A and GluN2B subunits, and AMPA receptors GluA1 subunits protein levels in the hippocampus. Furthermore, in baseline conditions EAAT3^{glo}/CMKII mice showed anxiety-like behavior in the open field test. Interestingly, mice with EAAT3 overexpression driven by CaMKIIα-promoter challenged to UCMS did neither show depressive-like behaviors nor impairment in sociability and memory.

Hippocampal glutamatergic system alterations may underlie the depressive-like behaviors. Moreover, we suggest that EAAT3 overexpression in the forebrain in mice may be linked to a resilient phenotype to chronic stress.

1. Introduction

1.1. Major depressive disorder

Major depressive disorder (MDD) affects approximately 5% of the worldwide population (World Health Organization, 2017). MDD is a debilitating mood disorder characterized by depressed mood, loss of interests and pleasure, impaired cognitive function and vegetative symptoms. According to the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5), an individual to be diagnosed with depression must show five or more of the symptoms described for MDD in the same 2-week period and including at least one of the core symptoms, which are depressed mood for most of the day and markedly diminished interest or pleasure in all, or almost all, activities (anhedonia). Other symptoms that may be experienced by depressed patients include change in weight or appetite, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or loss of energy, feelings of worthlessness or guilt, diminished ability to concentrate or indecisiveness, thoughts of death, recurrent suicidal ideation or a suicide attempt. The symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning. The depressive episode is not attributable to the physiological effects of a substance or to another medical condition (American Psychiatry Association, 2013). As for other neuropsychiatric disorders, decades of research on MDD has unveiled genetic and environmental factors; nevertheless, its etiology remains unclear.

1.2. Genetics of depression

Genetic epidemiology considers MDD a complex trait, meaning that multiple DNA sequence variations must be influencing risk in the population, along with nongenetic factors that interact with genes. Although MDD is the most common neuropsychiatric disorder in adults, it is the least heritable (Charney et al., 2017).

In a large-sample twin study, the heritability of MDD was estimated at 38%, and also evidencing that the heritability of liability to depression was significantly higher in women than men (Kendler et al., 2006). A family-based population study showed higher heritability for recurrent than single episode MDD, and a strong genetic correlation between early- and late-onset of depression. Also, in this study the heritability of MDD was estimated at 28% (Fernandez-Pujals et al., 2015). Even though these studies found a relevant genetic contribution to the etiology of MDD, to date no candidate genes have been found (Bosker et al., 2011), and no associated specific genetic variants have been significantly identified by genome-wide association studies (GWAS) (Sullivan et al., 2013; Levinson et al., 2014).

GWAS-based research has shown that psychiatric disorders are polygenic, with multiple common genetic variants, each in combination and with a summative effect that could be influencing more than one phenotype, suggesting an overlapping genetic etiology between psychiatric disorders (Mistry et al., 2018; Smeland et al., 2020). In a large trans-ancestry meta-analysis analyzing European and Han Chinese studies of MDD was identified a partially shared, common polygenic basis of MDD between these populations (Bigdeli et al., 2017). One replicated genome-wide significant locus associated with adult-onset MDD has been identified, and by polygenic score analysis it has been suggested that the genetic susceptibility to MDD differs between adult- and earlier-onset MDD, with earlier-onset cases having a greater genetic overlap with schizophrenia and bipolar disorder (Power et al., 2017). Nonetheless, another study found no age of onset MDD associated with polygenic risk for depression as measured by common genetic variants (Docherty et al., 2017). The shared heritability MDD with commonly comorbid psychiatric disorders could be one of the factors that influence the difficulty of finding a gene related to the susceptibility to manifest depression.

One of the assumptions of the multifactorial-polygenic model considers a single set of genetic and non-genetic risk factors for each MDD case (Levinson et al., 2014). This assumption could be generating difficulties in finding a candidate gene and single-nucleotide polymorphisms (SNPs) associations in MDD, because the heterogeneity present in mood disorders reduces both the statistical power as well as the observed risks attributed to susceptibility alleles or genotypes (Manchia et al., 2013). The lack of a robust and reliable biological marker for MDD diagnosis is a considerable factor that could contribute to the increase in heterogeneity in this type of research.

1.3. Hypothesis of depression

1.3.1. Monoamine hypothesis

It has been suggested that depression is caused by a deficiency in levels of one or more of the monoamines, including the indolamine serotonin (5-hydroxytryptamine, 5-HT), and the catecholamines norepinephrine (NE), dopamine (DA) and epinephrine.

In the midd-20th century, the monoamine hypothesis of depression was proposed after it was discovered that patients treated for hypertension with reserpine developed depression. The effects of this antihypertensive drug were associated with decreased levels of brain monoamines by blocking the neuronal vesicular monoamine transporter 2 (VMAT2), which inhibits uptake and reduces stores of the monoamine neurotransmitters in the synaptic vesicles of neurons, and non-stored neurotransmitters are metabolized by monoamine oxidases in the cytosol of the axon terminals (Schildkraut, 1965; Eiden and Weihe, 2011; Yaffe et al., 2018). As a result, reserpine increases removal of monoamine neurotransmitters from neurons, decreasing the size of the

neurotransmitter pools, and thereby decreasing the amplitude of neurotransmitter release. Moreover, at about the same time, imipramine, an experimental antihistamine with a tricyclic structure, and iproniazid, a drug used for the treatment of tuberculosis, were found to have antidepressant effects. Based on different mechanisms, both drugs were found to increase the extracellular levels of 5-HT and NE in the brain: imipramine by blocking their reuptake back to presynaptic endings, and iproniazid by inhibiting the enzymatic activity of monoamine oxidase (MAO) (Castrén, 2005), enhancing the effects of the neurotransmitters.

1.3.1.1. Serotonin

Evidence involving the serotonergic system in depressed patients includes the findings of both reduced 5-HT metabolites (Dencker et al., 1966; Owens and Nemeroff, 1994; Placidi et al., 2001) and its precursor, the amino acid tryptophan (Cowen et al., 1989). Individuals with one or two copies of the short allele of the serotonin transporter (SERT) promoter polymorphism have decreased SERT levels and enhanced susceptibility to exhibiting depressive symptoms in relation to stressful life events compared to individuals homozygous for the long SERT allele (Caspi et al., 2003; Clarke et al., 2010; Costafreda et al., 2013; Duman and Canli, 2015). However, GWAS-based research have found a poor association between SERT polymorphisms and MDD (Bosker et al., 2011). Moreover, anxiety-like behavior and hypolocomotion, but not depressive-like behaviors, have been observed in SERT-deficient mice (Lira et al., 2003; Kalueff et al., 2006, 2007).

Accumulating evidence also suggests a role for 5-HT receptors in the pathophysiology of depression. In MDD patients and suicidal cases, enhanced expression of the 5-HT_{1A} autoreceptors in the raphe nuclei has been associated with a polymorphism in the promoter region of the gene (Stockmeier et al., 1998; Lemonde et al., 2003). Positron emission tomography (PET) studies in MDD patients showed low 5-HT_{1B} receptor binding potential in the anterior cingulate cortex, hippocampus (Hpc) and ventral striatal/ventral pallidal region (Murrough et al., 2011; Tiger et al., 2016) and decreased 5-HT_4 receptor binding in the striatum (Madsen et al., 2015). In suicide victims diagnosed with depression, an altered editing of the mRNA encoding 5-HT_{2C} receptors in the prefrontal cortex (PFC) (Gurevich et al., 2002) and an increase in 5-HT_4 receptors in the frontal cortex and caudate nucleus (Rosel et al., 2004) were found.

In addition, preclinical studies in 5-HT_{1A} (Heisler et al., 1998), 5-HT_{1B} (Nautiyal et al., 2016), 5-HT_3 (Martin et al., 2017) and 5-HT_7 (Guscott et al., 2005) knockout (KO) mice exhibited antidepressant-like responses. Attenuated responses to stress were observed in a mouse model deficient in 5-HT_4 receptors (Compan et al., 2004).

1.3.1.2. Norepinephrine

Although the research into the pathophysiology of MDD related to the monoamine hypothesis have focused primarily on 5-HT signaling, there is vast evidence that points to a role of the noradrenergic system in depression. Noradrenaline genetic markers such as T182C polymorphism in the NE transporter (NET) gene have been associated with depressive disorders, although with opposite allele frequencies and genotypes related to susceptibility between Korean and Japanese populations studied (Inoue et al., 2004; Ryu et al., 2004). However, studies conducted in a European population (Zill et al., 2002) and in Chinese populations (Chang et al., 2007a; Cao et al., 2018) did not find an association between NET polymorphisms and MDD.

An increase in β-adrenergic receptor binding in the frontal cortex has been found in depressed suicide victims (Mann et al., 1986). Moreover, an upregulation of the α_{2A}-adrenoceptor has been observed in the frontal cortex and locus coeruleus from depressed patients (García-Sevilla et al., 1999; Ordway et al., 2003; Escribá et al., 2004). Additionally, downregulation of NET has been reported in the locus coeruleus of *postmortem* samples from MDD patients (Klimek et al., 1997). Furthermore, NET KO mice showed antidepressive-like behavior (Perona et al., 2008) and resistance to the stress-induced depression-like phenotype (Haenisch et al., 2009). On the contrary, α_{2A}-adrenoceptor-deficient mice were found to have increased stress-induced behavioral despair (Schramm et al., 2001).

1.3.1.3. Dopamine

Anhedonia, a core symptom seen in depression, is associated with dysfunctions in the DA system. Several investigations have revealed associations between susceptibility to depression and various DA neurotransmitter system, such as DA transporter (DAT) (Dong et al., 2009), tyrosine hydroxylase (TH) (Souery et al., 1996) and catechol-O-methyltransferase (COMT) (Funke et al., 2005). Studies on polymorphisms or genetic markers in DA receptors do not support a major role in contributing to susceptibility to MDD (Cusin et al., 2002; Hibino et al., 2006; Kõks et al., 2006).

Postmortem studies of the DA system in depressed patients demonstrated reduced DAT density and elevated D2/D3 receptor binding in the amygdala (Amy) (Klimek et al., 2002). Neuroimaging findings have showed reduced DAT (Meyer et al., 2001) and D1 receptor (Cannon et al., 2009), and increased D2 receptor (D'haenen and Bossuyt, 1994) binding potential in the striatum of depressed subjects. Preclinical studies have shown that DAT deficiency (Perona et al., 2008) and enhanced TH expression (Fu et al., 2006) elicit antidepressant-like effects in mice. Optogenetic selective inhibition and activation of ventral tegmental area (VTA) DA neurons induced depressive-like behaviors and rescued stress-induced depression-like phenotype in mice, respectively (Tye

et al., 2013). Moreover, the depression-related response to the inhibition or activation of VTA DA neurons depends on the specific projection targets (Chaudhury et al., 2013).

1.3.2. Neurotrophic hypothesis

This hypothesis proposes a key role for the reduction of neurotrophic factors in the vulnerability to manifest depression. Clinical and preclinical research in depression indicating volumetric loss in cortical and limbic brains regions in patients suffering from MDD and animal models of depression led to the emergence of the neurotrophic hypothesis of depression (Magariños et al., 1996; Bremner et al., 2000; Banasr et al., 2007; Zhao et al., 2017). Different classes of antidepressants increase trophic factors expression and signaling, which could induce hippocampal neurogenesis and reverse stress-induced changes in the brain (Malberg et al., 2000; Duman and Monteggia, 2006; Licznerski and Duman, 2013).

Most research has focused on brain-derived neurotrophic factor (BDNF). In humans, BDNF Val66Met polymorphism has been associated with poorer episodic memory, reduced activity-dependent BDNF release and impaired hippocampal function (Egan et al., 2003), and development of MDD as well (Verhagen et al., 2010; Hosang et al., 2014). In mice models of the Val66Met polymorphism impaired responses to antidepressants were found (Chen et al., 2006; Bath et al., 2012). Evidence based on preclinical studies does not reach consensus as to whether decreases in BDNF signaling induce depressive-like behaviors in mice due to the variability present in different studies (Lindholm and Castrén, 2014). Additionally, stress-evoked reduction or increase of BDNF is brain region-specific (Jaggar et al., 2019).

1.3.3. Gut microbiome hypothesis

The brain-gut-microbiota axis is a bidirectional communication system enabling gut microbes to communicate with the brain through the vagus nerve, short-chain fatty acids, cytokines, and tryptophan (Dinan and Cryan, 2017). In preclinical studies, chronic treatment with lactic acid bacteria reduced stress-induced deleterious effects and depressive-like behavior, and these effects were not found in vagotomized mice (Bravo et al., 2011). Moreover, in a maternal separation model in mice the presence of microbiota is required for the induction of behavioral despair (De Palma et al., 2015). Human studies have characterized the differences in the fecal microbiota composition in healthy subjects and MDD patients (Jiang et al., 2015; Chung et al., 2019).

1.4. Stress and hypothalamo-pituitary-adrenocortical axis

The hypothalamo-pituitary-adrenocortical (HPA) axis is required for stress adaptation (McEwen, 2007). Activation of the HPA axis is mediated by neurons localized in the medial parvocellular

portion of the hypothalamic paraventricular nucleus (PVN), and causes secretion of glucocorticoids (GCs), which act on multiple organ systems to redirect energy resources to cope real or anticipated demand. Homeostatic imbalance directly activates the PVN (Ulrich-Lai and Herman, 2009). Conversely, anticipatory HPA axis responses are indirectly mediated by limbic forebrain circuits (Herman et al., 2003). PVN neurons release corticotropin-releasing hormone (CRH) onto cells of the anterior pituitary inducing the secretion of adrenocorticotropin (ACTH) into systemic circulation. At the adrenal cortex, ACTH stimulates synthesis and release of GCs; cortisol in humans and corticosterone in rodents. Then, GCs can rapidly inhibit CRH release from PVN neurons by acting on membrane-associated receptors (Myers et al., 2012). In acute stress the feedback mechanisms effectively terminate the response after the stressor become extinct. In contrast, chronic stress generates changes in the basal activity of the HPA axis and in the responsiveness to stress (Herman et al., 1995). The HPA axis responds to each stressor, increasing the cumulative GCs burden. In addition, increased basal GC release triggered by chronic stress may be related to impairment of GC feedback control of the HPA axis (Herman et al., 2016).

1.5. Animal models in depression

Because the etiology of human depression is highly heterogeneous, modeling depressive disorders in animals is a complex challenge. In addition, symptoms such as feelings of guilt and sadness, depressed mood and suicide cannot be modelled in animals (Cryan and Holmes, 2005). Despite this, the risk of developing depression due to stress episodes is the basis of animal models of depression (Kessler, 1997; O'Leary and Cryan, 2013).

1.5.1. Validity criteria

The validity of an animal model is usually assessed through three classical criteria: predictive validity, face validity and construct validity (Planchez et al., 2019). Predictive validity is based on a pharmacological correlation of therapeutic results in humans which should be replicated in animals. The effects of chronic, but not acute, antidepressant administration is effective in reversing depressive-like behaviors in rodents is a good example of predictive validity (Nollet et al., 2013). Face validity refers to the phenomenological similarities between the human condition and the animal model. This criterion is evaluated by comparing the symptoms between the human condition and alterations of specific behavioral endpoints resulting from experimental manipulations related to the animal model of MDD (Belzung and Lemoine, 2011). Face validity attempts to simulate the diagnostic criteria of the psychiatric condition. For example, if anhedonia is considered one of the core symptoms of depression is, then a behavioral test should be used to assess anhedonia in the animal model of depression. Construct validity corresponds to the

equivalence of the triggering factors of the pathological condition (i.e. genetic vulnerability and environmental factors), and of the underlying neurobiological mechanisms (Planchez et al., 2019). For example, in the UCMS model in rodents, as in human depression, the depressive phenotype results from environmental and psychological stressors.

1.5.2. Behavioral models that trigger depressive-like behaviors

Behavioral paradigms that induce depressive phenotypes in animals have good predictive validity and attempt to model behavioral and biological domains of human depression.

1.5.2.1. Learned helplessness

This model aims to model one of the most common sentiments that patients suffering depression report, the feeling of diminished survival effort due to previous exposure to uncontrollable aversive stimuli (Pryce et al., 2011). In this behavioral paradigm, animals are placed in an closed chamber and receive several electric shocks on their feet repeatedly without the ability to escape the shock. After several conditioning periods, the animal is given opportunity to push a lever to stop the shock or given access to a neutral chamber allowing the animals to leave the aversive compartment (Vollmayr and Gass, 2013). Rodents that have not been previously exposed to the unescapable shock are commonly able to escape quickly from the shock, whereas animals previously exposed to the learned helplessness paradigm frequently fail to acquire shock avoidance.

1.5.2.2. Chronic social defeat stress

Chronic social defeat stress (CSDS) consists of subjecting defeated rodents to the home cage of an aggressive rodent on a daily basis. Physical contact between the two rodents is allowed for 5 – 10 min and then sensory contact for 24 h. This paradigm of depression has the advantage of not all rodents become susceptible to daily psychosocial stress. A percentage of defeated animals are resilient, assessing as a behavioral endpoint the social interaction with an unknown aggressive rodent (Golden et al., 2011). In addition to the validity of the construct, due to the individual differences of each individual to manifest a depressive phenotype, CSDS allows to study resilience mechanisms.

1.5.2.3. Unpredictable chronic mild stress

UCMS is a paradigm commonly used to induce depressive-like behaviors. Variations of this behavioral model are referred as chronic mild stress, chronic unpredictable stress, chronic unpredictable mild stress, and chronic variable stress, although essentially describe the same process (Willner, 2017). Daily exposure at least four weeks of stress exposure is capable of reliably inducing maladaptive phenotype (Monteiro et al., 2015). Generally, animals are exposed to one or

two stressor per day in a randomized fashion. Common stressors include disturbances in light cycle, cage flooding, stroboscope light, restraint, cage tilting, and shaking (Burstein and Doron, 2018). The importance of keeping the stressors unpredictable and randomized is crucial, because controllability or expectation of a stressor can attenuate UCMS-induced maladaptive behaviors, and usually results in anhedonia and reduced self-care (Nollet et al., 2013).

1.6. Neurocircuitry in depression

Main depressive symptoms, including long-lasting low mood and anhedonia, reflect predominant features of dysfunctional emotion regulation and reward processing. Neuroimaging studies in depression have shown structural and functional abnormalities in neural circuits involved in implicit emotion regulation, centered on the Amy and different regions in the medial PFC (mPFC), and reward neural circuitry, relied on the ventral striatum and mPFC (Phillips et al., 2015). The most reported volumetric abnormalities in MDD patients have been reduction of gray matter in the anterior cingulate cortex and PFC early in illness and in young adults at high familial risk for MDD (Price and Drevets, 2010), and this decrease is correlated with persistence of illness (Savitz and Drevets, 2009).

Recently, using PET with a radioligand targeting the synaptic vesicle glycoprotein 2A (SV2A) to estimate synaptic density, it was found that individuals with high levels of depression exhibited lower SV2A density in the PFC, anterior cingulate cortex and Hpc compared to controls, as well as impaired functional connectivity among these areas (Holmes et al., 2019). Additionally, *postmortem* studies of depressed subjects reported synaptic loss in the PFC, including lower expression of synaptic-function–related genes (Kang et al., 2012). In animal models, it has been observed that chronic stress – a widely used model of depression – decreases spine density and dendrite complexity of mPFC neurons (Li et al., 2011; Voleti et al., 2013).

In addition to the role in memory and learning processing, the Hpc has also been linked to the regulation of emotions. Imaging studies revealed greater functional connectivity of the Amy with the Hpc during negative stimuli recalling in depressed patients (Hamilton and Gotlib, 2008). Studies based on meta-analytic techniques suggest that hippocampal volume reductions generally occur after disease onset in MDD patients (McKinnon et al., 2009). Moreover, hippocampal neurogenesis is necessary for the antidepressant effects of chronic fluoxetine treatment in mice (Santarelli et al., 2003). Multiple studies have proposed that anhedonia, one of the core symptoms of depression, is linked to reward processing dysfunction, conceptualizing that reward comprises several constructs including motivation, salience, anticipation, prediction, learning, pleasure, and satiety (Heshmati and Russo, 2015). The reward circuit consists mainly of dopaminergic input from the VTA to the nucleus accumbens (NAc). The mesolimbic DA circuit

predicts and values the reward (Berridge and Robinson, 1998; Schultz, 1998). Nonetheless, neural reward processing is not unique to the VTA – NAc dopaminergic circuit. A role for the Amy in reward assessment has also been proposed (Murray, 2007). In addition, optogenetic inhibition of glutamatergic projections from the basolateral Amy (BLA) to NAc in mice reduced cue-induced sucrose intake, demonstrating an important role for this specific pathway in controlling reward-seeking behaviours (Stuber et al., 2011). Moreover, optogenetic stimulation of mPFC had antidepressant effects, and increased NAc activity associated with reversed social avoidance behavior in mice susceptible to social defeat (Covington et al., 2010). However, other studies have shown that attenuation of ventral Hpc (vHpc) – NAc glutamatergic transmission by optogenetic is pro-resilient and, conversely, acute enhancement of this input is pro-susceptible, whereas optogenetic stimulation of either mPFC or Amy afferents to the NAc is pro-resilient (Bagot et al., 2015). These observations suggest that glutamate, among other possible neurotransmitters involved, may play a key role in reward processing in the cortical-limbic circuit.

1.7. Memory and depression

In addition to deficits in emotional state, mood and enjoyment, depression also commonly affects attention, memory, and executive functions (Millan et al., 2012; McIntyre et al., 2015). In this regard, the DSM-5 identifies cognitive disturbances as symptoms of depression, such as difficulty thinking and concentrating or making decisions among the diagnostic criteria for MDD (American Psychiatry Association, 2013). These cognitive impairments are characterized by sudden onset, memory disorders, rapid progression, and self-report and emphasis of these deficits by patients (Perini et al., 2019). These signs tend to persist even during remission of depressive symptoms (Conradi et al., 2011). On the other hand, a rapid remission of depression symptoms is related to cognitive improvements experienced by patients (Gudayol-Ferré et al., 2015). Moreover, chronic antidepressant treatment in animal models of depression prevented (Bondi et al., 2008) and reversed (Danet et al., 2010) stress-induced cognitive deficits.

It is well known that stress is one of the factors that is involved in the etiology of mood disorders, and can have different consequences in the acquisition, consolidation, and retrieval of information. Stressful experiences may affect learning and memory processes, although both enhancing (Domes et al., 2002; Smeets et al., 2007) and impairing (Elzinga et al., 2005; Vogel and Schwabe, 2016) effects have been reported. The impact of stress on memory depends on multiple factors, such as the magnitude and intensity, timing, duration and controllability of the stressors. Acute stress frequently facilitates the consolidation of information, however impaires retrieval (Roozendaal, 2002; Joëls et al., 2006). Conversely, exposure to chronic stress can have negative

effects on decision-making, behavioral flexibility, and memory (Roozendaal, 2002; Myers et al., 2012; Wirkner et al., 2019).

Neuroimaging-based human studies have reported reduced PFC and hippocampal volumes in patients suffering from stress-related disorders (Bremner et al., 2000; Bremner, 2002; Lindauer et al., 2006; Lupien et al., 2007). Chronic stress in animal models impaired both structural and functional plasticity in the PFC (Radley et al., 2004, 2006; Zheng et al., 2011; Shansky and Lipps, 2013) and Hpc (Alfarez et al., 2003; Conrad, 2008; Krugers et al., 2010). Depressed patients with reduced Hpc volume showed deficits in memory tasks (Hickie et al., 2005). In MDD patients and animal models of depression were found dysfunction on Hpc-PFC circuit linked to memory deficits (Cerqueira et al., 2007; Genzel et al., 2015).

Several studies show that impairments to memory and cognitive performance could be related to dysregulation in glutamate neurotransmission. Mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1) displayed depressive-like behaviour and impaired recognition memory (Tordera et al., 2007). Loss of NMDA receptors GluN2A subunit (Brigman et al., 2008; Marquardt et al., 2014) and conditional deletion of the NMDA receptors GluN2B subunit on the forebrain (Brigman et al., 2010; Thompson et al., 2015) led to defective behavioral flexibility and memory in mice. Moreover, uncontrollable stress and blockade of NMDA receptors in the dorsal Hpc affect recognition memory in rats (Baker and Kim, 2002). Furthermore, detrimental effects on recognition memory, associated to reduced AMPA receptors- and NMDA receptors-mediated synaptic transmission and glutamate receptor expression were found in the PFC pyramidal neurons in chronic stress animal models (Yuen et al., 2012). Furthermore, acute subanesthetic-dose administration of ketamine reversed deficits in cognitive flexibility and depressive-like behavior in chronically stressed rats (Jett et al., 2015), which suggests the participation of NMDA receptors in mediating stress-evoked cognitive dysfunctions.

Alterations in EAATs have consequences on memory and cognitive performance. SNPs of EAAT1 and EAAT2 have been associated with déficits in working memory and cognitive functions in patients with psychiatric disorders (Spangaro et al., 2012; Poletti et al., 2014, 2015). Inhibition of EAATs with TBOA disrupts spatial memory retrieval in mice (Tak et al., 2007). Both pharmacologically induced EAAT2 upregulation and EAAT2 blockade impaired novel object recognition in rodents (Matos-Ocasio et al., 2014; Tian et al., 2019). In addition, a study focused on determining age-related cognitive decline mechanisms in mice reported that astrocytic EAAT2 deletion led to early deficits in spatial learning reference short- and long-term memory, and neuronal EAAT2 loss resulted in late-onset spatial reference long-term memory deficit (Sharma et al., 2019). Moreover, aged EAAT3 KO mice showed impairment in spatial memory related to

reduced glutathione content, brain atrophy and oxidative stress in the Hpc (Aoyama et al., 2006). By contrast, the cognitive improvement in learning and spatial memory as well as the increase of neuronal glutathione content and neuroprotection against oxidative stress could be associated with upregulation of EAAT3 levels in GTRAP3-18-deficient mice (Aoyama et al., 2012). However, the role of EAAT3 overexpression in object recognition and object location memory in mice under chronic stress conditions remains uncertain.

1.8. Glutamatergic system in depression

1.8.1. Glutamate signaling and receptors

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) (Orrego and Villanueva, 1993). Glutamatergic projections participate in almost all circuits in the CNS, encompassing intracortical circuits, cortical-subcortical connections, and subcortical systems such as the basal ganglia, cerebellum, thalamus, and brainstem circuits (Pittenger et al., 2011).

Glutamate binds on ionotropic and metabotropic receptors (Reiner and Levitz, 2018). Ionotropic glutamatergic receptors include AMPA, kainate, and NMDA receptors. These receptors are cation-permeable ion channels and function by allowing the influx of sodium and calcium ions into the cell, thereby generating depolarization and other intracellular changes (Kew and Kemp, 2005). NMDA receptors are heteromeric assemblies comprised of three types of subunits: GluN1, GluN2 and GluN3. There are eight different GluN1 subunits, four GluN2 subunits (A, B, C and D) and two GluN3 subunits. Functional expression of NMDA receptors must be composed of at least one GluN1 subunit and one GluN2 subunit (Paoletti and Neyton, 2007). NMDA receptors require binding of glycine in GluN1 subunits and binding of glutamate in GluN2 subunits to be activated (Furukawa et al., 2005). Besides, NMDA receptors are blocked by Mg^{2+} ions at resting membrane potentials. A depolarization of sufficient amplitude and duration is required to dislodge and repel the Mg^{2+} ions from the pore, thereby allowing the flow of permeant ions (Clarke et al., 2013). AMPA receptors are tetrameric ion channels composed of four types of subunits: GluA1, GluA2, GluA3 and GluA4, which combine in different stoichiometries to form distinct receptor subtypes (Greger et al., 2017). GluA2-containing AMPA recpetors are impermeable to calcium (Huganir and Song, 2002). Moreover, only two agonist molecules are necessary and sufficient for AMPA receptors activation (Clements et al., 1998).

Metabotropic glutamate receptors (mGluRs) are divided into Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3), and Group III (mGluR4 – 8). mGluRs are G-protein-coupled receptors (GPCRs). Group I mGluRs are coupled to G_q, whose activation increases phospholipase C (PLC) activity. Group II and Group III mGluRs are both are coupled to $G_{i/o}$, decreasing adenylyl cyclase levels (Crupi et al., 2019).

Antidepressant strategies development have also focusing on mGluRs. *Postmortem* studies showed elevated levels of mGluR2/3 (Feyissa et al., 2010), and decreased expresión levels of mGluR5 (Deschwanden et al., 2011) in the PFC of patients with MDD. Moreover, animal models of depression showed a decrease in mGluR2/3 (Matrisciano et al., 2008; Wierońska et al., 2008), and mGluR5 (Iyo et al., 2010) expresión levels in the Hpc and PFC, respectively. Administration of a group III mGluRs agonist produced antidepressant-like effect in rats (Pałucha et al., 2004). In addition, mGluR1/5 (Tatarczyńska et al., 2001; Belozertseva et al., 2007) or mGluR2/3 (Chaki et al., 2004) antagonists exert antidepressant-like effects in behavioral despair tests in rodents. Furthermore, in repeated corticosterone-treated rats a mGluR2/3 antagonist dampened the increased immobility time during the forced swimming test (Koike et al., 2013). Likewise, the pharmacological blockade of mGluR2/3 for one week reduced significantly the escape failures in the learned helplessness paradigm in rats (Yoshimizu et al., 2006). In chronically stressed animal models of depression, a single administration with mGluR2/3 antagonists produced rapid and sustained antidepressant effects (Dwyer et al., 2013; Dong et al., 2016). Nevertheless, six-week treatment with negative allosteric modulators of the mGluR2/3 (Umbricht et al., 2020) or mGluR5 (Quiroz et al., 2016) were not clinically effective in MDD patients.

Recent preclinical and clinical studies suggest that the glutamate neurotransmitter system may be involved in the pathogenesis of depression (Pittenger et al., 2007; Sanacora et al., 2012; Murrough et al., 2017). Elevated brain glutamate levels have been reported in depressive patients (Sanacora et al., 2008). Furthermore, MDD patients show a positive correlation between increased glutamate levels and the severity of depression (Mitani et al., 2006). Other studies have demonstrated that chronic treatment with antidepressants decrease reduce the serum levels of glutamate in depressed individuals (Maes et al., 1998). Moreover, ketamine, a NMDA receptor antagonist that is usually used as an anesthetic agent, has been shown to decrease symptoms of depression in patients with treatment-resistant depression within 2 h postinfusion, and continued to remain significant for 1 week (Zarate et al., 2006). However, the use of ketamine can induce side effects in patients (Zarate et al., 2006; aan het Rot et al., 2010). In preclinical studies, chronic exposure to corticosterone has been found to decrease the NMDA receptors GluN2B subunits and AMPA receptors GluA2/3 subunits in the mPFC of rats (Gourley et al., 2009). Besides, repeated stress decreases the total and surface levels of AMPA receptors GluA1 and GluA2 subunits and NMDA receptors GluN1, GluN2A and GluN2B subunits in the mPFC of rats (Yuen et al., 2012). Furthermore, chronic stress leads to maladaptive changes within glutamate synapses, including reduced extracellular glutamate clearance and the increased activation of extrasynaptic NMDA receptors GluN2B subunits (Murrough et al., 2017). Antagonists selective for NMDA receptors GluN2B subunits reverse depressive-like behaviors in an animal model of depression in mice

(Maeng et al., 2008). Collectively, these studies suggest that dysregulation of glutamatergic signaling is associated with depressive disorders.

1.8.2. Excitatory amino acid transporters

Once glutamate is released into the synaptic cleft, excitatory amino acid transporters (EAATs) control the diffusion and glutamate content in the extracellular space (Vandenberg and Ryan, 2013), providing a fine-tuning transmission and protecting against excitotoxicity (Bridges and Esslinger, 2005). In the CNS there are five isoforms of EAATs, EAAT1 and EAAT2, localized in glial cells (Lehre et al., 1995; Shigeri et al., 2004), EAAT3 expressed in dendrites and cell bodies of glutamatergic, GABAergic, dopaminergic and interneuron postsynaptic neurons in the forebrain (Danbolt, 2001; Amara and Fontana, 2002; Holmseth et al., 2012), EAAT4 in Purkinje cells of the cerebellum (Itoh et al., 1997; Dehnes et al., 1998), and EAAT5 in the retina (Pow and Barnett, 2000). Glutamate transport mediated by EAAT1, EAAT2, and EAAT3 is coupled to the cotransport of three equivalents of sodium ions and one proton followed by the countertransport of one equivalent of potassium ions. On the other hand, the transport stoichiometry of EAAT4 and EAAT5 has not been determined. In addition, there is a chloride flux coupled to glutamate transport, but this differs between the different subtypes of EAATs. EAAT4 and EAAT5 function predominantly as chloride channels, whereas for EAAT1, EAAT2 and EAAT3 the chloride flux is in a lower proportion than the ion fluxes associated with transporter function (Vandenberg and Ryan, 2013).

1.8.3. EAAT3

Immunostaining studies indicate that EAAT3 is preferentially expressed in perisynaptic region of excitatory synapses, localized immediately beside the synaptic specialization space, thus regulating glutamate spillover (He et al., 2000; Waxman et al., 2007). Despite the glutamate uptake process is carried out mostly by the astrocytic transporters EAAT1 and EAAT2 (Holmseth et al., 2012), studies indicate that EAAT3 plays a key physiological role in the clearance of glutamate, and to regulate the responses of extrasynaptic AMPA and NMDA receptors (Scimemi et al., 2009; Underhill et al., 2014; Delgado-Acevedo et al., 2019). Additionally, EAAT3 controls GABAergic neurotransmission by supplying glutamate as a substrate for GABA synthesis in GABAergic neurons (Mathews and Diamond, 2003).

Changes in the expression levels of EAAT3 have been related to the development of depressive-like behaviors. In a prenatal stress model in rats, a decrease in EAAT3 mRNA levels was observed in the Hpc (Zhang et al., 2013). Furthermore, early-life stress induced a decrease in EAAT3 expression levels in the vHpc and in the cerebral cortex of rats (Kim et al., 2020). In the same study, EAAT3 KO mice exhibited depressive-like behaviors. Moreover, administration of ketamine to chronically stressed rats alleviated depressive-like behaviors and reversed the stress-induced

decrease in EAAT3 expression levels, and the increase in extracellular glutamate in the Hpc (Zhu et al., 2017). These findings indicate a possible correlation between EAAT3 expression levels and depressive-like behaviors, and suggest that EAAT3 downregulation may contribute to the manifestation of a depressive phenotype.

1.9. Summary

As mentioned above, in preclinical and clinical studies of depression, an increase in glutamate and dysregulation of glutamatergic signaling in cortical and limbic brain areas have been observed. Furthermore, in chronic stress animal models, both decreased glutamate uptake and reduced expression of EAATs, including EAAT3, were observed. Due to its perisynaptic localization in postsynaptic neurons, EAAT3 might play a key role in protecting from overactivation of extrasynaptic glutamate receptors and preventing an aberrant glutamatergic signal associated with maladaptive changes that could lead to a depressive phenotype. Altogether, the evidence leads to the hypothesis that upregulation of EAAT3 levels in the cortical-limbic brain circuit could promote resilience to develop depressive-like behaviors in an animal model of depression.

1.10. Hypothesis

Genetically-induced increased EAAT3 expression in the cortical-limbic circuit confer a resilient phenotype in the acquisition of the depressive-like phenotype triggered by chronic stress.

1.11. General goal

To understand the role of EAAT3 in depressive-like behaviors and the consequences of chronic stress on the glutamate transporters and receptors in the cortical-limbic brain areas.

1.12. Specific aims

- To evaluate the consequences of UCMS in WT mice at the behavioral level, using a battery of memory tests and depressive-related behavioral paradigms.
- To determine the consequences of UCMS on the glutamate transporters and ionotropic receptors expression levels in the cortical-limbic brain areas in WT mice,using western blot determinations.
- To evaluate the impact of EAAT3 overexpression in the forebrain of mice in the UCMS model at the behavioral level, using a battery of memory tests and depressive-related behavioral paradigms.
- To evaluate the consequences of CSDS in WT mice at the behavioral level, using a battery of depressive-like behaviors.

2. Materials and methods

2.1. Animals

Animals were male and female WT, and EAAT3 overexpressing (EAAT3^{glo}/CMKII) and control (EAAT3^{glo}, no Cre) mice on a C57/BL6J background strain. The animals were experimentally naïve and 2 – 6 months old (25 – 30 g). Animals were weaned at 21 days of age, and group-housed (3–5 animals per cage) in an animal facility room with controlled temperature (21 ± 2°C) and humidity (40-70%), with *ad libitum* food and water, in a 12 h light:dark cycle (lights on at 8:00 AM). All experiments were performed blind to the animal genotype.

2.1.1. Mouse lines with conditional EAAT3 overexpression in the forebrain

Thesis advisor, Dr. Pablo Moya, generated a line of transgenic mice with conditional EAAT3 overexpression in the forebrain by Cre/LoxP system, using a pCLE-EAAT3 vector (Fig. 1A) as described Delgado-Acevedo et al., 2019. The main elements of this vector include a constitutive CMV/β-actin promoter, followed by an enhanced green fluorescent protein (EGFP) sequence, which contains a 3' polyA tail as a stop sequence. EGFP is flanked by LoxP regions and a mouse EAAT3 cDNA construct is located downstream. pCLE-EAAT3 vector was extensively characterized in vitro (Fig. 1B and C), and subsequently used to generate the EAAT3^{glo} mice (Fig. 1D).

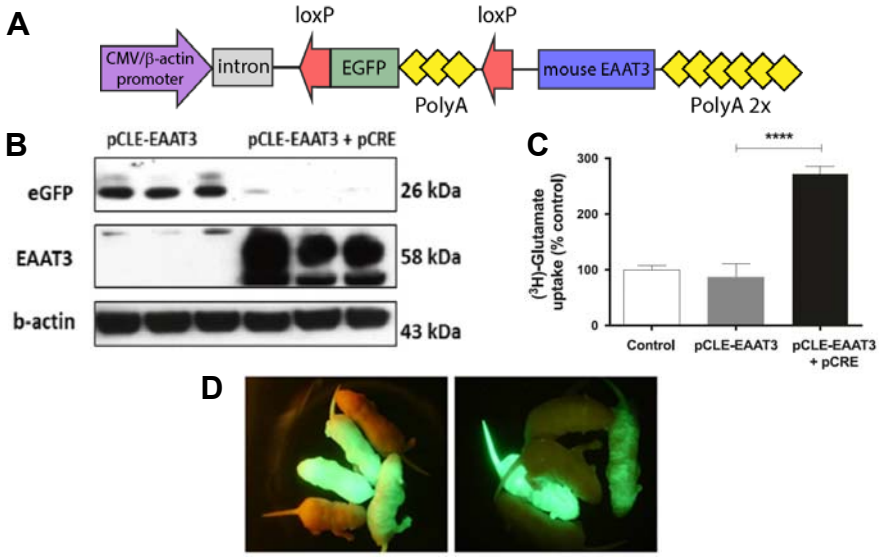

Figure 1. Characterization of pCLE-EAAT3 vector. The pCLE-EAAT3 vector contains a floxed EGFP gene sequence. Upon Cre recombination, the EGFP gene is released and EAAT3 is expressed **(A)**. Representative immunoblots for GFP, EAAT3, and beta-actin expression from N2A cells transfected with pCLE-EAAT3, pCLE-EAAT3, and pCMV-Cre during 24 h **(B)**. [^{3}H]-Glutamate uptake determinations in N2A cells transfected with pCLE-EAAT3, pCLE-EAAT3, and pCMV-Cre during 24 h **(C)**. EAAT3^{glo} mice visualized under UV light **(D)**.

To obtain mice with conditional EAAT3 overexpression in neurons in the forebrain we crossed EAAT3^{glo} mice with CaMKIIα-Cre mice. This is transgenic mouse line promotes Cre/LoxP recombination under the control of the promoter of the alpha-calcium/calmodulin-dependent protein kinase II (CaMKIIα) gene (Tsien et al., 1996). Thus, in the presence of Cre recombinase, EGFP and the stop sequence is deleted and EAAT3 is conditionally expressed (Fig. 2).

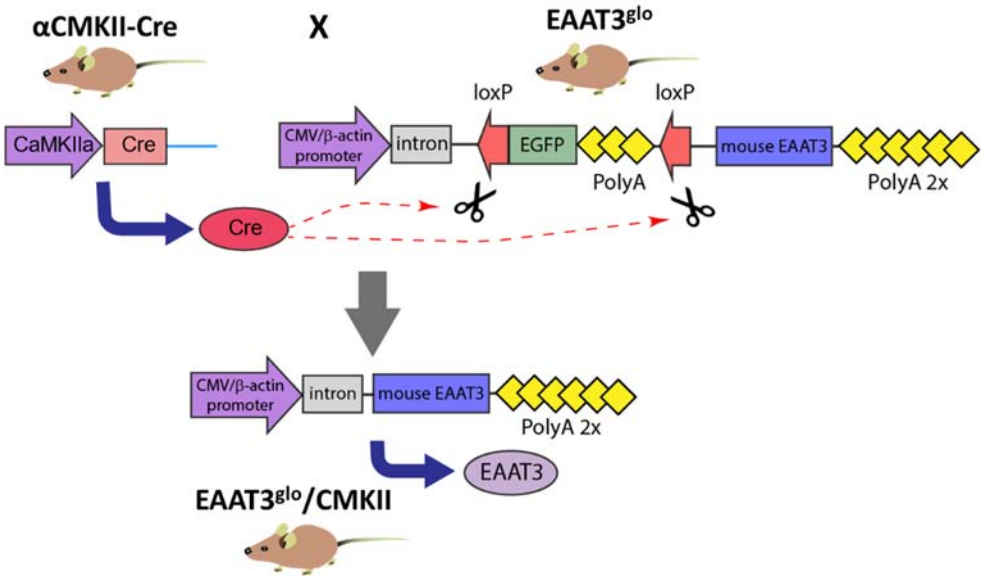

Figure 2. Scheme of crosses to obtain conditional EAAT3 overexpression mice restricted to CaMKIIα expressing neurons in the forebrain.

2.2. Genotyping

2.2.1. Standard PCR

Our lab developed a triplex PCR assay for GFP (presence or absence of EAAT3 transgene), CRE (presence or absence of CaMKIIα-Cre), and MAO-B (internal control). Primers for promoter-to-GFP region were PROM-For: 5′-CTCTAGAGCCTCTGCTAACC-3′; EGFP-Rev: 5′-TGATGCCGTTCTTCTGCTTGTC-3′, 346 bp amplicon. CRE primers were CRE-For: 5′-GCATACCTGGAAAATGCTTCTGT-3′; CRE-Rev: 5′-GGCCCAAATGTTGCTGGATAGTT-3′, amplicon 164 bp. MAO-B primers were MAO-B-For: 5′-CTACAAAGCAGATTGCCACGC-3′; MAO-B-Rev: 5′-TACCTGACATCAACTGGTCCC-3′, amplicon 292 bp. 2 µl of DNA, 8 µl of primer set (0.4 µM final concentration), and 10 µl 2X Sapphire (RR350A, Takara Bio USA, Inc, Mountain View, CA, USA) were mixed in each tube. Cycle: 95°C 10 min; [94°C 30 s, 62°C 30 s, 72°C 30 s] X 30 cycles; 72°C 10 min. Amplicons were run on 2% agarose electrophoresis in TBE buffer (pH 8.4).

In DNA samples from WT mice a single 292 bp band was observed, corresponding to internal control MAO-B (Fig. 3, lane 2). In EAAT3^{glo} control mice, two bands were observed: 346 bp corresponding to EAAT3 transgene and 292 bp internal control MAO-B (Fig. 3, lane 3). In CaMKIIα-Cre mice, 2 bands were observed: 292 bp corresponding to internal control MAO-B and 164 bp of Cre Recombinase (Fig. 3, lane 4). In DNA samples from mice with conditional EAAT3 overexpression in principal neurons in the forebrain (EAAT3^{glo}/CMKII), three bands were observed: 346 bp corresponding to the EAAT3 transgene, 292 bp internal control MAO-B, and 164 bp Cre recombinase PCR product (Fig. 3, lane 5).

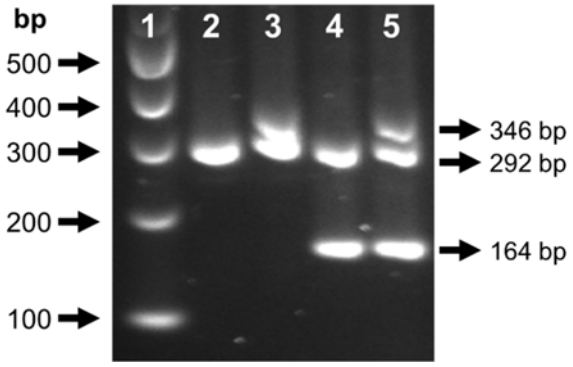

Figure 3. Agarose gel electrophoresis with amplicons obtained by standard PCR. Lane 1: 100 bp DNA ladder (#SM0323, Thermo Fisher Scientific Inc, Waltham, MA, USA). Lane 2: WT, lane 3: EAAT3^{glo}, lane 4: CaMKIIα-Cre, and lane 5: EAAT3^{glo}/CMKII mouse.

2.2.2. Genomic DNA extraction

Genomic DNA was extracted from a 0.5 cm long tail segment of each of the mouse litters obtained. In 400 µl of lysis buffer (0.1 M Tris, 0.2 M NaCl, 5 mM EDTA and 0.4% SDS) supplemented with proteinase K (Sigma Aldrich Inc., St. Louis, Missouri, USA) 0.2 mg/ml the tail samples were incubated at 55°C until complete disintegration of the tissues. The disaggregated tissues were centrifuged at 13000 rpm for 3 min at room temperature. 500 µl of pure isopropanol (Sigma Aldrich Inc., St. Louis, Missouri, USA) were added to each supernatant. The samples were centrifuged again at 13000 rpm for 3 min at room temperature. The supernatants were discarded, and 1 ml of 70% ethanol (ethanol for molecular biology, Sigma Aldrich Inc., St. Louis, Missouri, USA) were added to the pellet. Following vortex, the tubes were centrifuged again at 13000 rpm for 3 minutes at room temperature. The supernatants were discarded and the pellet inside the tubes were left to dry at room temperature for 10-15 minutes. Then, 100 µl of sterile double distilled water prewarmed at 55°C were added to each tube and the tubes were incubated in shaking at 37°C for 10 minutes or until the DNA pellet was completely resuspended. DNA was quantified in a spectrophotometer, obtaining the sample purity ratio (260 nm/280 nm) ranging from 1.7 to 1.9.

2.3. Behavior

Animals were transferred to the behavioral room in their home cages, acclimated for at least 1 h and tested between 10:00 and 16:00 h (during the light cycle). Equipment was cleaned after each mouse with ethanol 70% to eliminate odor cues. All tests and analyses were done blind to the genotype of animals.

At the beginning of the experiments, mice of all genotypes were randomly divided into two groups (Table 1): non-stressed control (baseline conditions) and another group subjected to UCMS.

Table 1. Number of individuals corresponding to each genotype and treatment.

Genotype	Group	n
WT	Control	10
WT	UCMS	10
EAAT3^{glo}	Control	15
EAAT3^{glo}	UCMS	14
EAAT3^{glo}/CMKII	Control	9
EAAT3^{glo}/CMKII	UCMS	7

2.3.1. Unpredictable chronic mild stress

Mice were daily subjected to one or more stressor according a semi-random schedule for 5 weeks (Table 2). The stressors used consisted of restraint stress, alterations of the light and dark cycle, wet bedding, substitution of sawdust with water, removal of sawdust, tilting the cage by 45°, repeated changes of bedding, replace home cage bedding by the bedding of a CF-1 mouse and stroboscope light (Table 3).

Over the 5 weeks of stress, body weight and coat state were evaluated twice a week in all animals. Following the UCMS protocol, WT, EAAT3^{glo} and EAAT3^{glo}/CMKII mice were tested in anxiety- and depressive-like behaviors, and compared to their respective non-stressed control groups (Fig. 4). Mice were stressed once a day over the behavioral testing period.

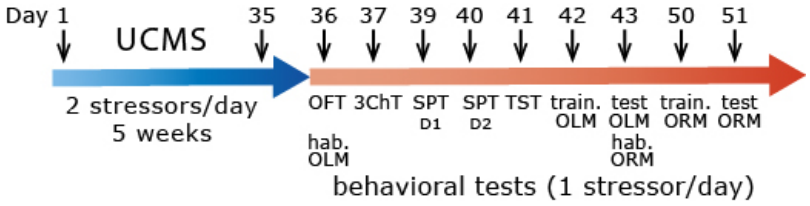

Figure 4. Scheme of experimental UCMS timeline. At the end of the 5 weeks of UCMS, behavioral tests in non-stressed control and stressed animals were initiated. Open field test (OFT), three-chamber social interaction test (3ChT), sucrose preference test (SPT), tail suspension test (TST), training (train.), object location memory (OLM), and object recognition memory (ORM). Body weight and coat state were measured twice a week during UCMS.

Table 2. 5-week schedule of UCMS.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1	BW+Coat state Light + No bedding (overnight)	Restraint (1h, AM) Bedding CF-1 mouse (5h, PM)	Restraint (1h, PM) No bedding (overnight)	BW+Coat state Dark (5h, AM) Cage tilting (3h, PM)	Shaking (1h, AM) Light (overnight)	Restraint (1h, AM) Wet bedding (overnight)	Water (2h, PM) Light (overnight)
Week 2	BW+Coat state Cage tilting (4h, AM) Water (2h, PM)	Cage tilting (2.5h, PM) Light (overnight)	Restraint (1h, AM) Wet bedding (overnight)	BW+Coat state Dark (5h, AM) Stroboscope (overnight)	Cage tilting (2.5h, AM) Dark (6h, PM) Light (overnight)	No bedding (5h, AM) Stroboscope (overnight)	Dark (5h, AM) Cage tilting (4h, PM)
Week 3	BW+Coat state Restraint (1h, AM) Shaking (1h, PM)	Dark (5h, AM) Water (2h, PM)	No bedding (5h, AM) Stroboscope (overnight)	BW+Coat state Restraint (1h, AM) Light (overnight)	Cage tilting (2.5h, PM) Water (2h, PM)	Restraint (1h, AM) Shaking (1h, PM)	No bedding (5h, AM) Stroboscope (overnight)
Week 4	BW+Coat state Shaking (1h, AM) Light (overnight)	Cage tilting (2.5h, PM) No bedding (4h, PM)	No bedding (5h, AM) Water (2h, PM)	BW+Coat state Restraint (1h, PM) Light (overnight)	Cage tilting (3h, PM) Stroboscope (overnight)	Dark (5h, AM) Light (overnight)	Bedding CF-1 mouse (5h, PM) Stroboscope (overnight)
Week 5	BW+Coat state Dark (5h, AM) Water (2h, PM)	Restraint (1h, AM) Wet bedding (overnight)	No bedding (5h, AM) Stroboscope (overnight)	BW+Coat state Cage tilting (2.5h, PM) Light (overnight)	Cage tilting (2.5h, AM) Dark (6h, PM) Light (overnight)	Shaking (1h, AM) Light (overnight)	Cage tilting (4h, AM) Water (2h, PM)

Body weight (BW), morning (AM), afternoon/evening (PM).

Table 3. Experimental stressors.

Stressor	Description
Light/Dark cycle disturbances	Mice cages were placed in a dark/light testing room during normal light/dark hours.
Wet bedding	Bedding was moistened with the addition of approximately 100 mL of water.
No bedding	All bedding and enrichment were removed from home cage.
Restraint	The mice were kept in closed and ventilated tubes (12 cm length × 5 cm i.d.). Mice had the possibility to turn themselves back into the tube.
Bedding CF-1	The home cage bedding was replaced by the bedding of a CF-1 mouse.
Cage tilting	The cages were tilted backwards (45 degrees).
Shaking	Groups of five mice were placed in a plastic box container and placed in an orbital shaker at 150 rpm.
Water	The bedding of each cage was removed and replaced by about 125 ml water at 20◦C (about 1 cm water).
Stroboscope light	Mice cages were placed in a dark testing room and exposed to a medium intensity stroboscope light.

2.3.2. Coat state

The coat state of each animal was measured on seven body parts of the mouse: head, neck, back, abdomen, tail, forepaws and hindpaws (Fig. 5). The coat state score resulted from a qualitative scoring for each body part, a score 0 was given for good state (smooth and shiny fur), a score 0.5 was given for moderate degradation (fur with no tousled, and some spiky patches) and a score 1 for bad coat state (fluffy fur on with slight staining). The total score was obtained by the sum of the scores for all seven body parts, with a maximum possible score of 7. Thus, scale from 0 to 7, means good to bad.

2.3.3. Body weight gain

Body weight gain was calculated by subtracting the body weight measured twice a week over the UCMS protocol minus the initial weight of the mice prior to exposure to stress (week 0).

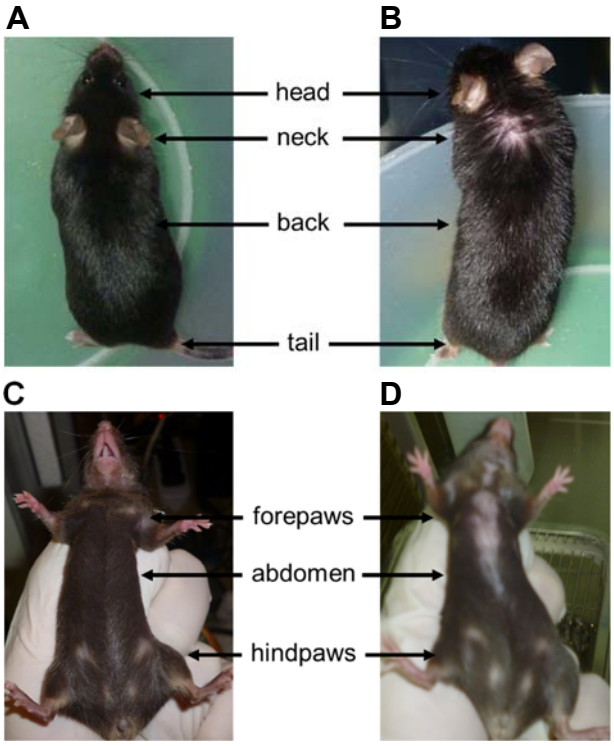

Figure 5. Representative images of fur condition. Non-stressed control mouse **(A** and **C)** and UCMS-subjected mouse **(B** and **D)**. The coat state score resulted from a qualitative assessment of different parts of the body including the head, the neck, the forepaws, the back, the abdomen, the hindpaws, and the tail.

2.3.4. Open field test

An individual mouse was introduced to the center in an open field arena (40 x 40 x 35 cm^3) into a procedural room dimly lit by indirect red lighting (~50 lux). During 5 min, the total distance traveled (cm), time (s) of permanence or exploration in the center (20 x 20 cm^2) or in the periphery (5 cm from the walls of the arena), and number of entries to center were recorded for each individual. Between animals, the arena was cleaned with 70% ethanol, to remove olfactory cues. Mouse behavior was recorded and analyzed using the Noldus Ethovision Video Tracking System (Version 7.0; Noldus, Wageningen, Netherlands).

2.3.5. Sucrose preference test

Sucrose preference test was performed in stressed groups on day 4 after UCMS. Each mouse was removed from the home cage and place it individually in a cage filled with fresh sawdust with *ad libitum* food and, in addition, for non-stressed control mice, nesting material for enrichment. Mice were habituated to two identical drinking bottles with water for 48 h. After acclimatization, mice were presented to two drinking bottles: one containing 1% sucrose solution and the other

water for 2 days in their home cage. The positions of two bottles were changed on the second day to reduce place preference. Volume intake of water and sucrose were measured once per day. Sucrose preference was calculated as a percentage of sucrose intake over the total volume of fluid intake (Eq. 1).

$$\%\ \text{sucrose preference} = \left(\frac{V_{sucrose}}{V_{water} - V_{sucrose}} \right) x\ 100$$

Equation 1. Calculation of percentage for sucrose preference. $V_{sucrose}$ and V_{water}, volume of sucrose and water consumed, respectively.

2.3.6. Tail suspension test

Each subject was suspended by its tail from a bar 60 cm above the floor using adhesive tape. Ambient white lighting (~300 lux) was present in the room throughout testing. The mouse was positioned such that it had no contact with other objects. To prevent mice from climbing their tails, clear hollow cylinders (3 cm length, 1.6 cm outside diameter, 1.3 cm inside diameter, 1.5 grams) were placed around the tails of mice. The trials were conducted for a period of 6 min and were video recorded. The behavioral measure was the duration of immobility in the last four minutes, interpreted as behavioral despair.

2.3.7. Three chambered social interaction test

A clear acrylic rectangular three-chambered apparatus was used to assess sociability. Each of the three cameras measured 20 cm (length) × 40.5 cm (width) × 22 cm (height) divided by transparent walls with small openings which have removable doors by the experimenter to control access to the chambers. Lighting was adjusted keeping dim red light (~10 lux) uniform throughout the apparatus. Target mice (used as a social novelty) were trained to the inverted wire pencil cup inside a side chamber of the maze. Training included three 10-minute sessions. The sociability test consists of three stages. First, the test session began with habituation of experimental mouse for 10 min, only in the central chamber, with the doors closed, without access to the side chambers. In the next stage, the doors separating the central chamber from lateral chambers were opened to allow the experimental mouse to explore and habituate to all three chambers for 10 min. The subject was then briefly confined to the central chamber while the experimenter placed an empty inverted wire pencil cup (novel object) in one of the lateral chamber, and a unfamiliar target mouse (social novelty) under a inverted wire pencil cup in the other lateral chamber. A small white bottle of 20 grams was placed on the top of each inverted wire pencil cup, to prevent the subject mouse from climbing on top. After both wire cups were positioned, the two doorways were simultaneously opened and the subject was allowed access to all three chambers for 10 min. The apparatus and cups were cleaned with 70% ethanol between subjects. Time spent sniffing by the subject to the

empty cup and cup containing the target mouse was recorded. Interaction ratio was calculated based on time spent sniffing the target mouse divided by the total time sniffing the target mouse plus the time sniffing the empty cup (Eq. 2). Exploring time of each camera was obtained using Noldus Ethovision Video Tracking System (Version 3.0; Noldus, Wageningen, Netherlands).

$$\text{interaction ratio} = \frac{t_{mouse}}{t_{mouse} - t_{object}}$$

Equation 2. Calculation of social interaction ratio. t_{mouse} and t_{object}, time spent sniffing cup with target mouse and empty, respectively.

2.3.8. Depression score

Depression score (Eq. 3) was calculated as the number of tests (N) minus the algebraic sum of standardized scores for each of the three main analyzed parameters of the three depression-related behavioral tests (sucrose preference test (sucrose preference (%)), tail suspension test (mobility time (s)) and three-chamber social interaction test (time spent sniffing the novel mouse (s)). Standardization consisted in subtracting the value of each animal to the minimum value of the whole population and then dividing this number by the maximum value of the whole population minus minimum value of the whole population: (x – min value)/(max value – min value). This procedure yields scores which are distributed along a scale from 0 to 3, 3 reflecting high depression.

$$\text{depression score} = N - \sum_{i=1}^{N} \left(\frac{x - min_{val}}{max_{val} - min_{val}} \right)$$

Equation 3. Expression to calculate depression score. N, number of depression-related behavioral tests; x, analyzed parameters; min_{val} and max_{val}, minimum and maximum value of the whole population, respectively.

2.3.9. Object location and object recognition memory tests

First, the animals were handled 1 – 2 min for 5 days and then habituated to the object location memory (OLM) experimental apparatus (square arena, 40 x 40 x 35 cm^3, with ~1 cm depth of bedding to the floor, and the light adjusted between 45 – 48 lux) for 5 min once a day for 6 days in the absence of objects. On training day, mice were placed into the experimental apparatus with two identical objects and allowed to explore for 10 min. After 24 h of the training trial, in the OLM test, only one of the objects was moved from location relative to the training session (Fig. 6A), and mice were allowed to explore the experimental apparatus for 5 min. Exploration was scored when the head of a mouse was oriented toward the object within a distance of 1 cm or when the nose was touching the object. The relative exploration time was recorded and expressed as a discrimination index (DI), calculated as 100 multiplied by the quotient between the difference of

time spent exploring displaced object less the time spent exploring nondisplaced object, divided by total time exploring both objects (Eq. 4).

$$\text{OLM DI} = \frac{t_{\substack{\text{displaced}\\\text{object}}} - t_{\substack{\text{nondisplaced}\\\text{object}}}}{t_{\substack{\text{displaced}\\\text{object}}} + t_{\substack{\text{nondisplaced}\\\text{object}}}} \times 100$$

Equation 4. Expression to calculate OLM DI.

Subsequently, mice were habituated to the object recognition memory (ORM) experimental apparatus (circular arena, 28,5 cm in diameter x 23 cm high, with ~1 cm depth of bedding to the floor, and the light adjusted between 45 – 48 lux) for 5 min once a day for 6 days in the absence of objects. In training session animals were presented with two identical objects for 10 min (Fig. 6B). Twenty-four hours later, mice were places into ORM arena for 5 min, in which one of the ORM training objects was replaced with a novel, previously unexplored object. The relative exploration time was analyzed for total exploration of objects in addition to the ORM DI, calculated as 100 multiplied by the quotient between the difference of time spent exploring novel object less the time spent exploring familiar object, divided by total time exploring both objects (Eq. 5).

$$\text{ORM DI} = \frac{t_{\substack{\text{novel}\\\text{object}}} - t_{\substack{\text{familiar}\\\text{object}}}}{t_{\substack{\text{novel}\\\text{object}}} + t_{\substack{\text{familiar}\\\text{object}}}} \times 100$$

Equation 5. Expression to calculate ORM DI.

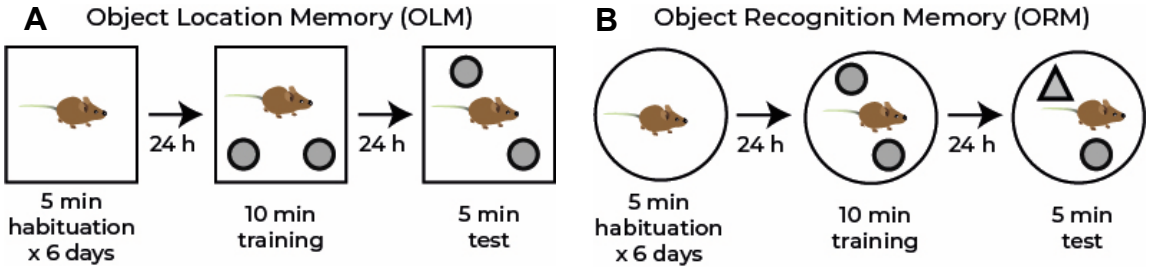

Figure 6. Object location and object recognition memory task design. Experimental timeline for OLM **(A)** followed by ORM **(B)**. During testing, for OLM the left object is shown as the displaced object, and for ORM the left object is shown as the novel object.

2.3.10. Chronic social defeat stress

CF-1 mice used for all defeat experiments were previously screened for consistent attack latencies (<30 sec on 3 consecutive screening sessions with a C57BL/6J intruder). Non-defeated controls were housed in identical cages opposite each other and were rotated similarly. C57BL/6 WT mice were subjected to episodes of social defeat for 21 consecutive days (Fig. 7). Each day, an intruder C57BL/6J mouse was introduced for 5 – 10 min in the home cage of an aggressive and unknown CF-1 mouse. After physical interaction, both mice were kept in sensory contact separated by a transparent acrylic sheet.

2.3.11. CF-1 social interaction test

On day 22, after CSDS, in an open field arena (40 x 40 x 35 cm^3) into a procedural room dimly lit by indirect red lighting (~ 50 lux), the time spent by defeated mice in the interaction zone was recorded during two tests. The first test with a wire-mesh enclosure with target CF-1 mouse absent and the second with CF-1 mouse present inside the wire-mesh enclosure. Each 2.5 min session was carried out consecutively. Between the 2 sessions, the defeated mouse was removed from the arena, and placed back in its home cage for approximately 1 min. CF-1 social interaction ratio was calculated as (interaction time with CF-1 mouse present) / (interaction time with CF-1 mouse absent).

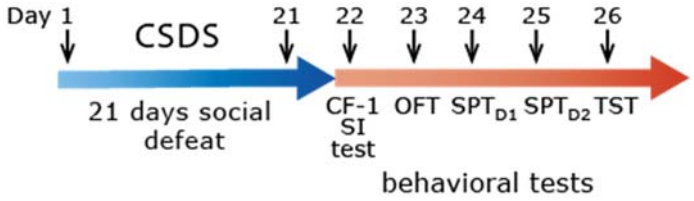

Figure 7. Scheme of experimental CSDS timeline. At the end of the 21 weeks of CSDS, behavioral tests in non-stressed control and stressed animals were initiated. CF-1 SI test (CF-1 social interaction test), open field test (OFT), three-chamber social interaction test (3ChT), sucrose preference test (SPT) and tail suspension test (TST). Body weight was measured every two days during CSDS.

2.4. Western blot

Mice were killed by decapitation, and brains were immediately removed from the skull. After dissecting the mPFC, dorsal striatum (Str), NAc, and vHpc samples, these tissues were homogenized in RIPA lysis buffer containing protease inhibitors (#78430, Thermo Fisher Scientific Inc, Waltham, MA, USA) and phosphatase inhibitors (J63907, Alfa Aesar, Tewksbury, MA, USA). The homogenate was incubated at 4°C for 20 min, sonicated and centrifuged at 14,000 × g at 4°C for 30 min, and the supernatant was collected. The concentration of proteins was measured by the Bradford assay. Crude protein extracts (50 µg) were denatured and separated on a SDS-PAGE

gel at 8%. After separation, protein was transferred on PVDF membranes (Amersham GE Healthcare, Bucks UK). The blots were blocked in 5% non-fat milk in phosphate-buffered saline with 0.1% Tween-20 for 1 hour at room temperature and incubated overnight at 4°C using antibody against EAAT1 (1:5000; ab416, Abcam), EAAT2 (1: 500; sc-365634, Santa Cruz Biotechnology), EAAT3 (1:500; 12686-1-AP, Proteintech), anti-GluN2A (1:1000; 07-632, Merck-Millipore), anti-GluN2B (1:1000; BWJHL, Merck-Millipore), anti-GluA1 (1:1000; D4N9V, Cell Signaling Technology), Anti-GluA2 (1:1000; E1L8U, Cell Signaling Technology), and β-actin (1:5000; ab8227, Abcam). These blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies and detected by ECL Select reagent (Amersham GE Healthcare, Bucks UK). The density of the selected bands was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5. Statistical analysis

Statistical analysis was performed using Origin 9.0 software (OriginLab Corp). Data are expressed as mean values ± standard error of the mean (SEM), unless otherwise noted. All data were analyzed to determine if they fitted to normal distribution using the Shapiro-Wilk test. For normally distributed data, Student's t-test was perfomed. One-Way ANOVA , Two-Way ANOVA, and Two-Way Repeated Measures ANOVA, with Fisher post *hoc* analysis were perfomed for multiple groups comparisons. Probability (p) < 0.05 was considered statistically significant in all tests.

3. Results

3.1. Unpredictable chronic mild stress in WT mice

3.1.1. Body weight gain and coat state

We successfully established the UCMS model in the laboratory, following the protocol by Nollet et al., 2013 with modifications in the number of weeks of stress and type and order of stressors applied. UCMS significantly reduced the body weight gain in WT mice from week 1, which was maintained over time, as seen in Fig. 8A (Two-Way Repeated Measures ANOVA, $F(2.5, 45.7) = 10.56$, $p < 0.0001$). UCMS also induced a significant deterioration of the coat state, as demonstrated by increasing coat state scores (Fig. 8B; Two-Way Repeated Measures ANOVA, $F(4, 72) = 43.48$, $p < 0.00001$).

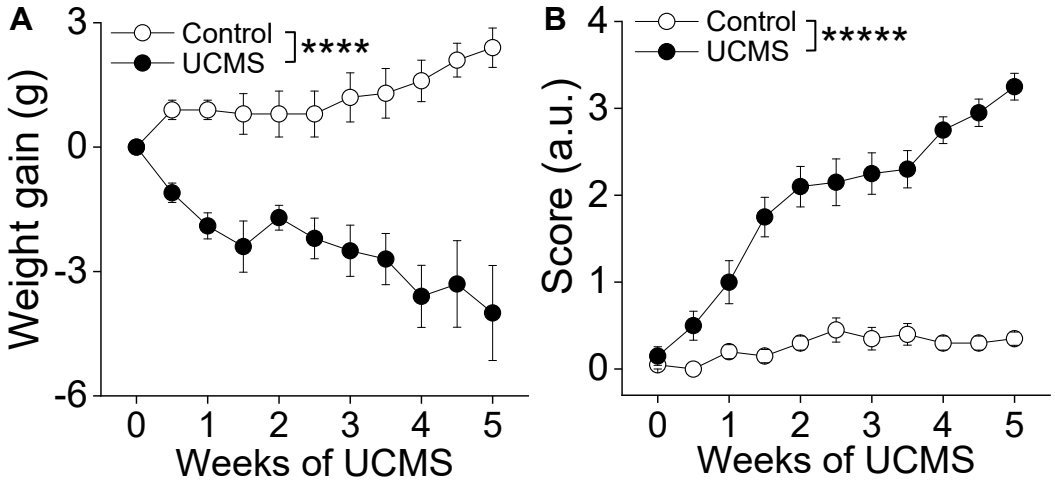

Figure 8. Effects of 5 weeks UCMS on the physical state of WT mice. The UCMS significantly disrupts the body weight gain **(A)**, and deterioration of the coat state **(B)**. Data are expressed as mean ± SEM; ****$p < 0.0001$ and *****$p < 0.00001$. Control n = 10 and UCMS n = 10.

3.1.2. Open field test

WT mice challenged to UCMS spent less time in center of the open field test (Fig. 9B; t-test = 8.2, df = 18, $p < 0.00001$) and showed reduction of entries to center (Fig. 9C; t-test = 7.6, df = 18, $p < 0.00001$), indicative of an anxiety-like behavior. No differences in total distance traveled (Fig. 9D; t-test = 0.65, df = 18, $p = 0.52$) were observed.

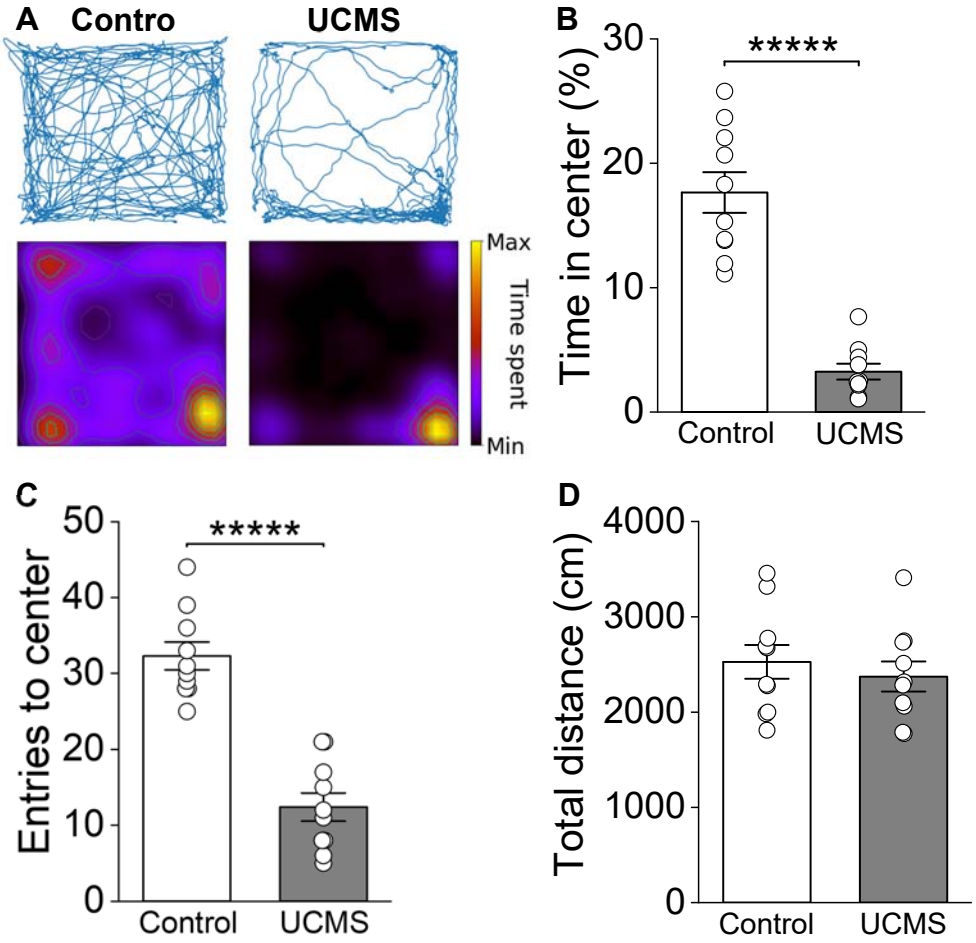

Figure 9. Effects of 5 weeks UCMS on anxiety-like behavior in WT mice. Representative linear (top) and heatmap (bottom) trackings in the open field test for each group **(A)**; yellow represents increased time spent and dark blue represents minimal time spent over trial. Stressed WT mice showed significant reduction in time in center **(B)** and frequency to visit the center of arena **(C)**. No significant differences were seen in total distance **(D)**. Data are expressed as mean ± SEM; *****$p < 0.00001$. Control n = 10 and UCMS n = 10.

3.1.3. Sucrose preference and tail suspension tests

UCMS caused a significant reduction in sucrose preference (Fig. 10A; t-test = 7.76, df = 18, $p < 0.00001$) and more time of immobility in tail suspension test (Fig. 10B; t-test = -5.77, df = 18, $p < 0.0001$). Therefore, UCMS resulted in a depressive-like behavior using these two classical paradigms relevant to depression.

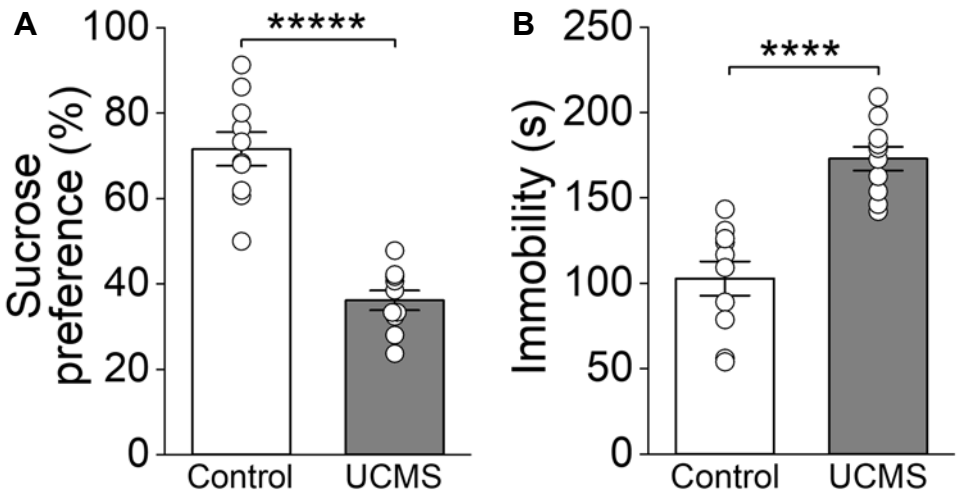

Figure 10. Effects of UCMS on depressive-like behavior in WT mice. Stressed WT mice showed significant reduction in sucrose consumption **(A)** and significantly increased time of immobility in tail suspension test **(B)**. Data are expressed as mean ± SEM; ****p < 0.0001. Control n = 10 and UCMS n = 10.

3.1.4. Three-chambered social interaction test

We then evaluated social behavior using the three-chambered social interaction test. Social behavior was measured as the interaction ratio (Eq. 2). WT mice subjected to chronic stress showed a significant decrease in the sociability measure as indicated in the social interaction ratio (Fig. 11; t-test = 5.20, df = 18, p < 0.001).

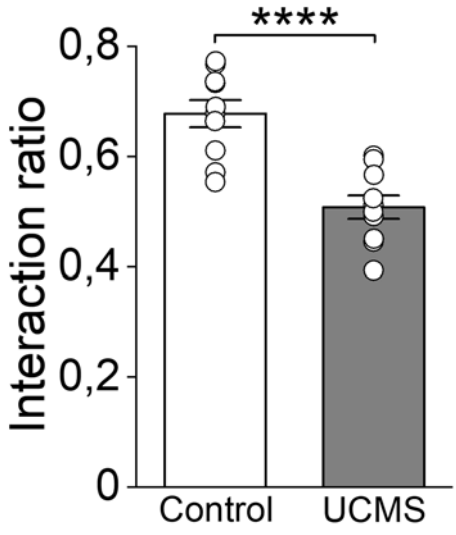

Figure 11. Effects of UCMS on social behavior in WT mice. Stressed WT mice showed significant reduction in social interaction in the three-chambered social interaction test. Data are expressed as mean ± SEM; ****p < 0.0001. Control n = 10 and UCMS n = 10.

3.1.5. Depression score

Depression score (Eq. 3) is used in order to get a global portrait of depressive-like behavior encompassing the sucrose preference test, tail suspension test and the three-chambered social interaction test data. This measure is distributed along a scale from 0 to 3, 3 reflecting high depression. WT mice challenged to the UCMS paradigm showed significantly higher depression scores (Fig. 12; t-test = -10.18, df = 18, $p < 0.00001$), reflecting overall depressive-like behavior.

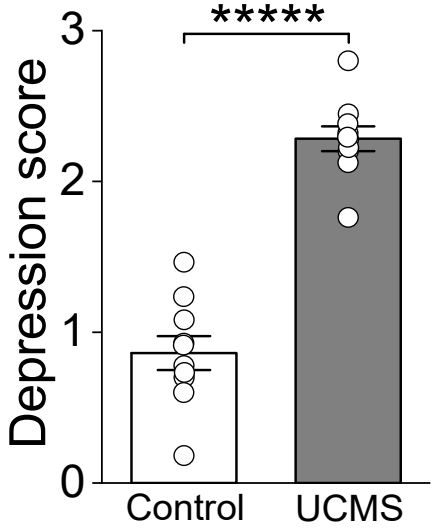

Figure 12. Effects of UCMS on global depression score in WT mice. Stressed WT mice showed showed higher depression score values. Data are expressed as mean ± SEM; *****$p < 0.00001$. Control n = 10 and UCMS n = 10.

3.1.6. Object location and object recognition memory tasks

Using OLM and ORM tasks we assessed the consequences of UCMS on episodic memory. WT mice chronically stressed spent less time exploring the object displaced in the OLM (Fig. 13E; t-test = 2.62, df = 10.53, $p < 0.05$) and the novel object in the ORM (Fig. 13F; t-test, t-test = 2.58, df = 5.75, $p < 0.05$).

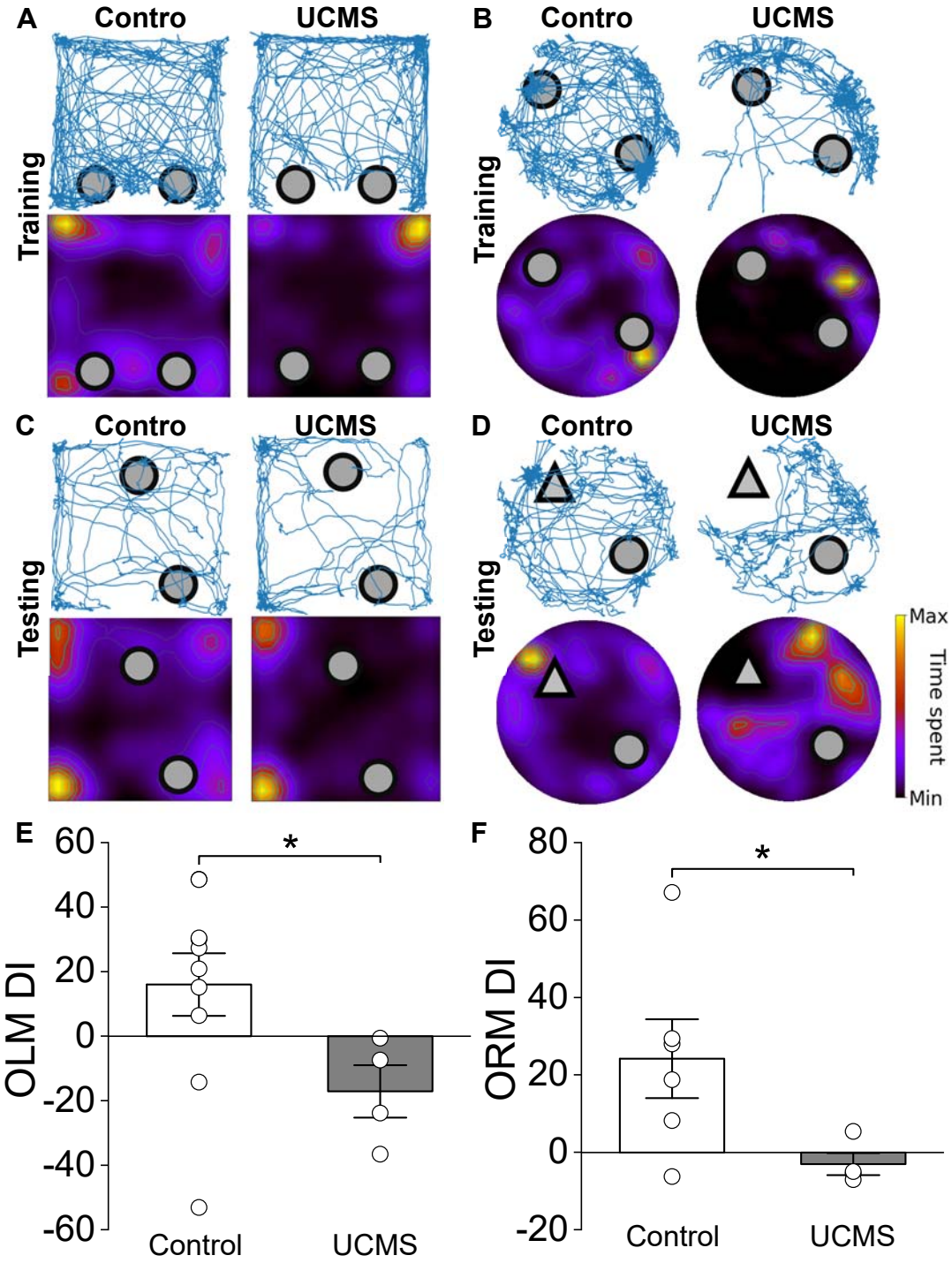

Figure 13. Effects of UCMS on OLM and ORM in WT mice. Representative linear (top) and heatmap (bottom) trackings in the OLM training **(A)** and testing **(C)**, and ORM training **(B)** and testing **(D)**, for each group; yellow represents increased time spent and dark blue represents minimal time spent over trial. Stressed WT mice showed showed lower OLM DI **(E)** and ORM DI **(F)**. Data are expressed as mean ± SEM; *p < 0.05. OLM: Control n = 10 and UCMS n = 4, and ORM: Control n = 6 and UCMS n = 4.

3.1.7. Western blot determinations

Samples of the mPFC, Str, NAc and vHpc from non-stressed and stressed WT mice groups were analyzed for protein levels of glutamate transporters EAAT1, EAAT2 and EAAT3, AMPA receptors subunits GluA1 and GluA2, and NMDA receptors subunits GluN2A and GluN2B.

There were no significant differences in EAAT1 protein levels in the mPFC (Fig. 14A; t-test = 0.48, df = 10, p = 0.64), Str (Fig. 14B; t-test = 0.23, df = 10, p = 0.82), and NAc (Fig. 14C; t-test = -1.15, df = 10, p = 0.28). Furthermore, stressed WT mice showed upregulation of EAAT1 protein levels in the vHpc (Fig. 14D; t-test = -2.61, df = 4, p = 0.05). No significant differences between non-stressed and stressed WT mice were found in EAAT2 protein levels in the mPFC (Fig.14E; t-test = 1.57, df = 4, p = 0.19), Str (Fig. 14F; t-test = -0.26, df = 4, p = 0.81), NAc (Fig. 14G; t-test = -1.09, df=4, p = 0.33) and vHpc (Fig. 14H; t-test = 1.03, df = 4, p = 0.36). and EAAT3 protein levels in the mPFC (Fig. 14I; t-test = 0.74, df = 4, p = 0.49), Str (Fig. 14J; t-test = 1.57, df = 4, p = 0.18), NAc (14K; t-test = 0.55, df = 10, p = 0.59) and vHpc (Fig. 14L; t-test = 0.99, df = 10, p = 0.35).

No differences in GluN2A protein levels in the mPFC (Fig. 15A; t-test = 0.61, df = 8, p = 0.55), Str (Fig. 15B; t-test = 0.13, df = 8, p = 0.89) and NAc (Fig. 15C; t-test = 0.68, df = 8, p = 0.51) were observed. WT mice challenged to UCMS showed an increase in GluN2A expression levels in the vHpc (Fig. 15D; t-test = -2.99, df = 8, $p < 0.05$). GluN2B protein levels did not change between non-stressed and stressed WT mice in the mPFC (Fig. 15E; t-test = -2.09, df = 10, p = 0.06), in the Str (Fig. 15F; t-test = -0.27, df = 10, p = 0.78) and NAc (Fig. 15G; t-test = 0.53, df = 10, p = 0.60). Moreover, an increase in GluN2B protein levels were observed in the vHpc of WT stressed mice (Fig. 15H; t-test = -2.57, df = 10, $p < 0.05$).

GluA1 protein levels in the mPFC (Fig. 16A; t-test = 0.60, df = 4, p = 0.58), Str (Fig. 16B; t-test = -1.73, df = 4, p = 0.15) and NAc (Fig. 16C; t-test = 0.57, df = 4, p = 0.59) showed no significant differences, whereas GluA1 expression levels were increased in the vHpc of stressed group (Fig. 16D; t-test = -5.21, df = 4, $p < 0.01$). GluA2 protein levels were similar between non-stressed and stressed WT mice in the mPFC (Fig. 16E; t-test = 1.43, df = 4, p = 0.22), Str (Fig. 16F; t-test = 0.27, df = 4, p = 0.79), NAc (Fig. 16G; t-test = 1.17, df = 4, p = 0.30) and vHpc (Fig. 16H; t-test = -2.33, df = 4, p = 0.08).

For an overview of the changes in protein expression in the brain areas studied in mice subjected to chronic stress, we plotted a protein expression profile on a colorimetric matrix (Fig. 17).

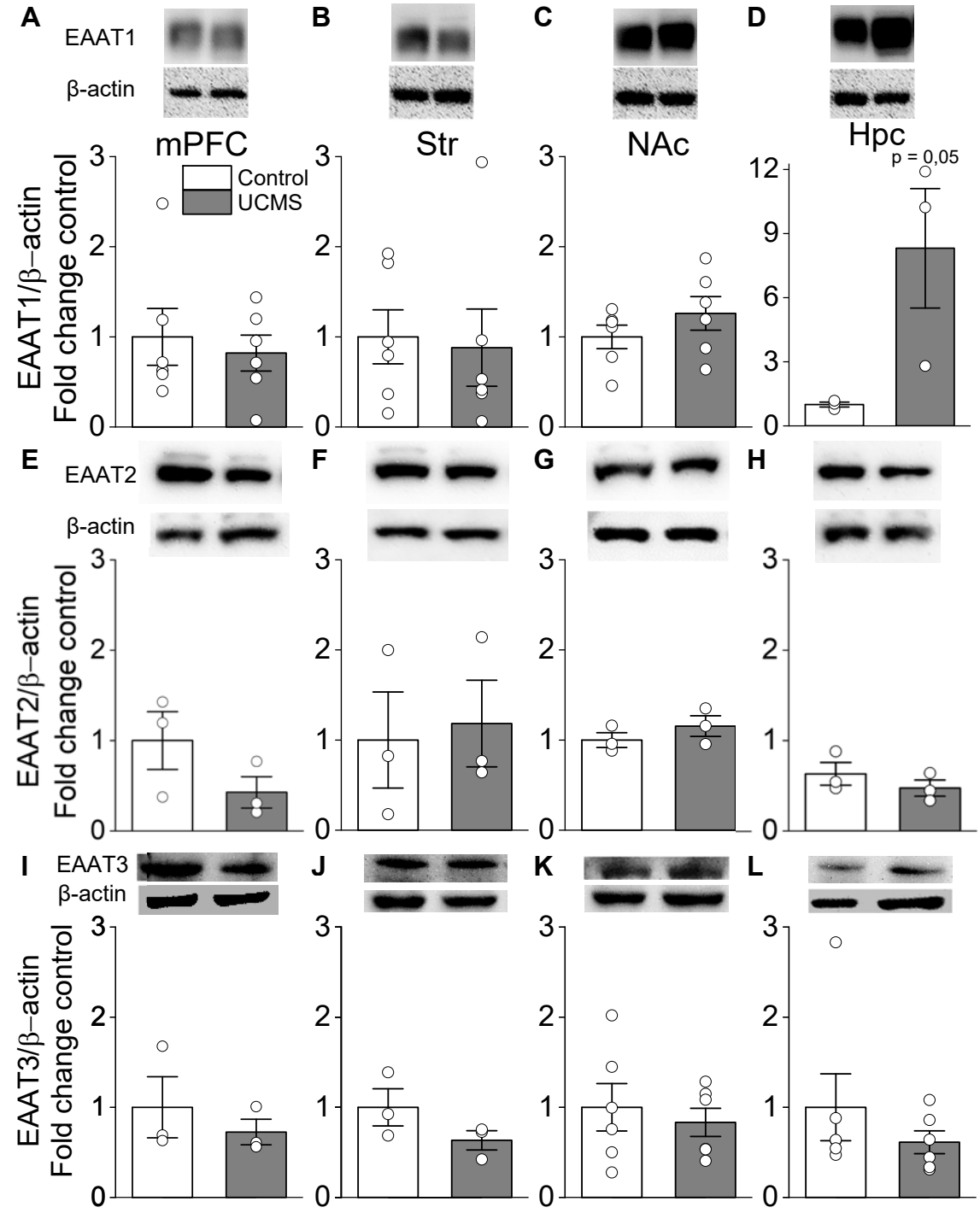

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Figure 14. Effects of UCMS on glutamate transporters expression levels in WT mice. No significant differences in EAAT1 (60 kDa) expression levels in mPFC **(A)**, Str **(B)** and NAc **(C)** were observed between non-stressed and stressed WT mice. In stressed WT mice increased EAAT1 protein levels in the vHpc **(D)**. No significant changes in EAAT2 (62 kDa) protein levels in mPFC **(E)**, Str **(F)**, NAc **(G)** and vHpc **(H)**, and in EAAT3 (57 kDa) protein levels in mPFC **(I)**, Str **(J)**, NAc **(K)** and Hpc **(L)** were observed in stressed WT mice. Protein expression levels were normalized to the β-actin (43 kDa) loading control and presented relative to the control group. Data are expressed as mean ± SEM. EAAT1 mPFC, Str and NAc, Control n = 6 and UCMS n = 6; Hpc, Control n = 3 and UCMS n = 3. EAAT2 Control n = 3 and UCMS n = 3. EAAT3 mPFC and Str, Control n = 3 and UCMS n = 3; NAc and Hpc, Control n = 6 and UCMS n = 6.

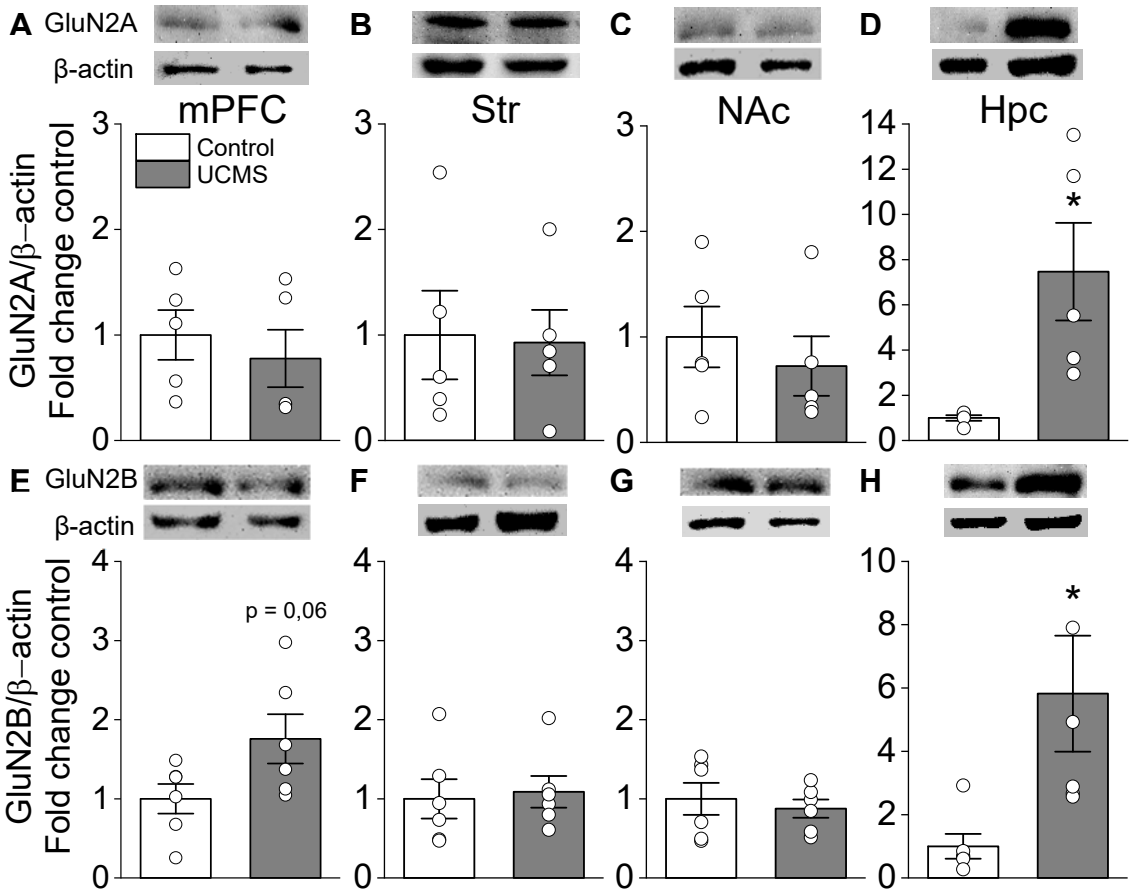

Figure 15. Effects of UCMS on NMDA receptors subunits expression levels in WT mice. No differences were observed in GluN2A (170 kDa) expression levels in mPFC **(A)**, Str **(B)** and NAc **(C)** between non-stressed and stressed WT mice. In stressed WT mice increased GluNRA protein levels in the Hpc **(D)**. No changes were observed in GluN2B (180 kDa) levels in mPFC **(E)**, Str **(F)** and NAc **(G)** between both groups. GluN2B levels increased in the Hpc of stressed WT mice **(H)**. Protein levels were normalized to the β-actin (43 kDa) loading control and presented relative to the control group. Data are expressed as mean ± SEM; *p < 0.05. GluN2A Control n = 5 and UCMS n = 5. GluN2B Control n = 6 and UCMS n = 6.

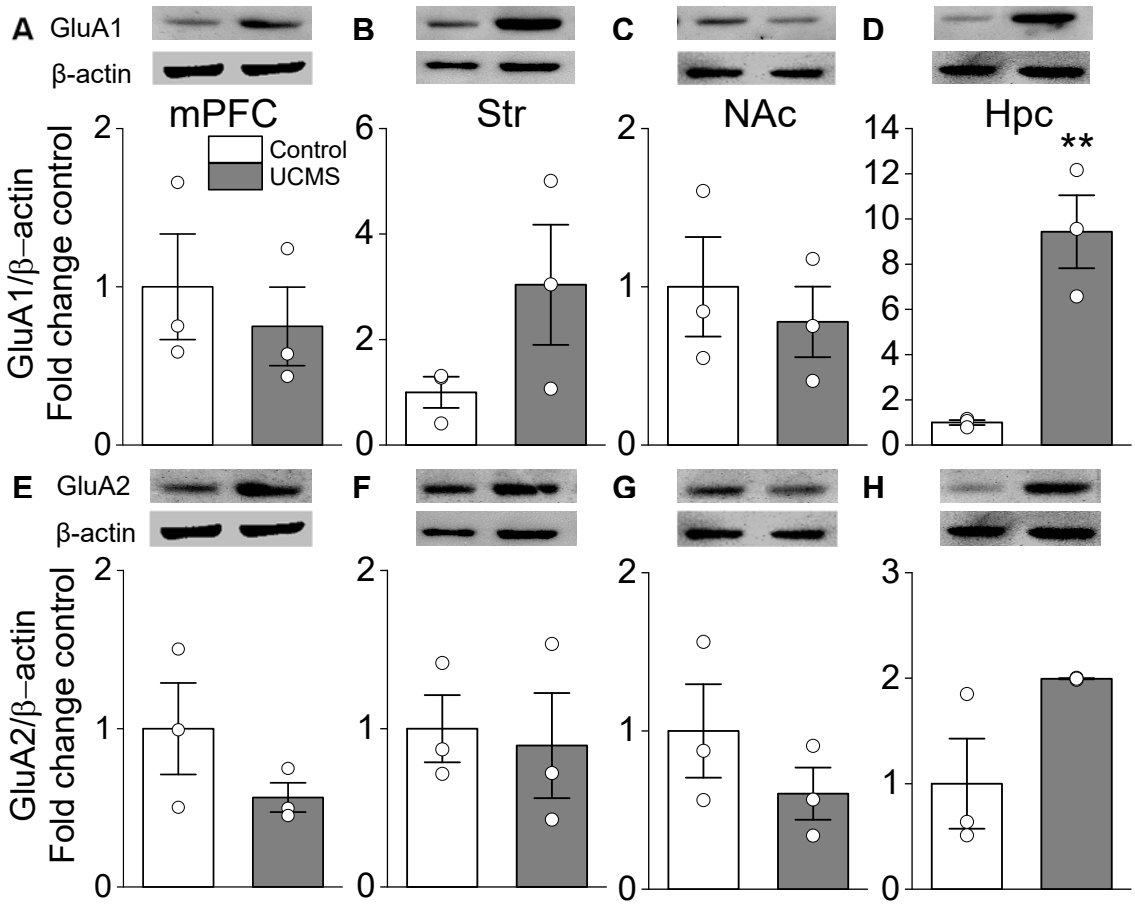

Figure 16. Effects of UCMS on AMPA receptors subunits expression levels in WT mice. No significant differences were observed in GluA1 (100 kDa) expression levels in mPFC **(A)**, Str **(B)** and NAc **(C)** between non-stressed and stressed WT mice. In stressed WT mice increased GluA1 protein levels in the vHpc **(D)**. No significant changes were observed in GluA2 (100 kDa) protein levels in mPFC **(E)**, Str **(F)**, NAc **(G)**, and vHpc **(H)** between both groups. Protein expression levels were normalized to the β-actin (43 kDa) loading control and presented relative to the control group. Data are expressed as mean ± SEM; **p < 0.01. GluA1 and GluA2 Control n=3 and UCMS n=3.

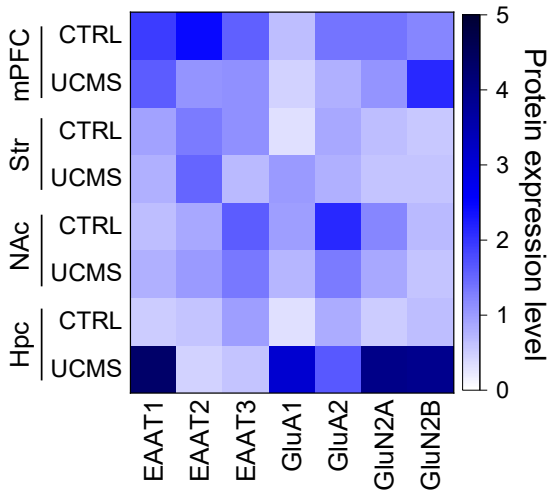

Figure 17. UCMS upregulated glutamate receptors in WT mice. In mice subjected to chronic stress, a significant increase in GluN2A ($n = 5$, $p < 0.05$), GluN2B ($n = 6$, $p < 0.05$) and GluA1 ($n = 3$, $p < 0.01$) protein levels were observed in the vHpc. Data are expressed as mean of protein expression levels normalized to the β-actin (43 kDa) loading control and presented relative to the control group.

3.2. Unpredictable chronic mild stress in mice with EAAT3 overexpression

We used a mouse model with an increase level in the expression of the neuronal glutamate transporter EAAT3 at excitatory synapses. This model, driven by CaMKIIα-promoter (EAAT3^{glo}/CMKII) was compared to the control group (EAAT3^{glo}) to evaluate alterations in anxiety- and depressive-like behaviors triggered by UCMS paradigms, as well as baseline levels.

3.2.1. Body weight gain and coat state

UCMS significantly reduced the body weight gain in EAAT3^{glo} mice from week 2, which was maintained over time, as seen in Fig. 18A (Two-Way Repeated Measures ANOVA, $F(6.6, 93.6) = 9.33$, $p < 0.00001$. *post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo} – UCMS, $t = 12.53$, $p < 0.00001$). Body weight gain was unaffected in chronically stressed EAAT3^{glo}/CMKII mice (*post hoc* Fisher test: EAAT3^{glo} – UCMS vs EAAT3^{glo}/CMKII – UCMS, $t = 7.40$, $p < 0.00001$). UCMS also induced a significant deterioration of the coat state in EAAT3^{glo} mice, as demonstrated by increasing coat state scores (Fig. 18B; Two-Way Repeated Measures ANOVA, $F(9.55, 133.75) = 12.04$, $p < 0.00001$). In EAAT3^{glo}/CMKII mice, UCMS did not significantly affect the coat state (*post hoc* Fisher test: EAAT3^{glo} – UCMS vs EAAT3^{glo}/CMKII – UCMS, $t = 18.97$, $p < 0.00001$).

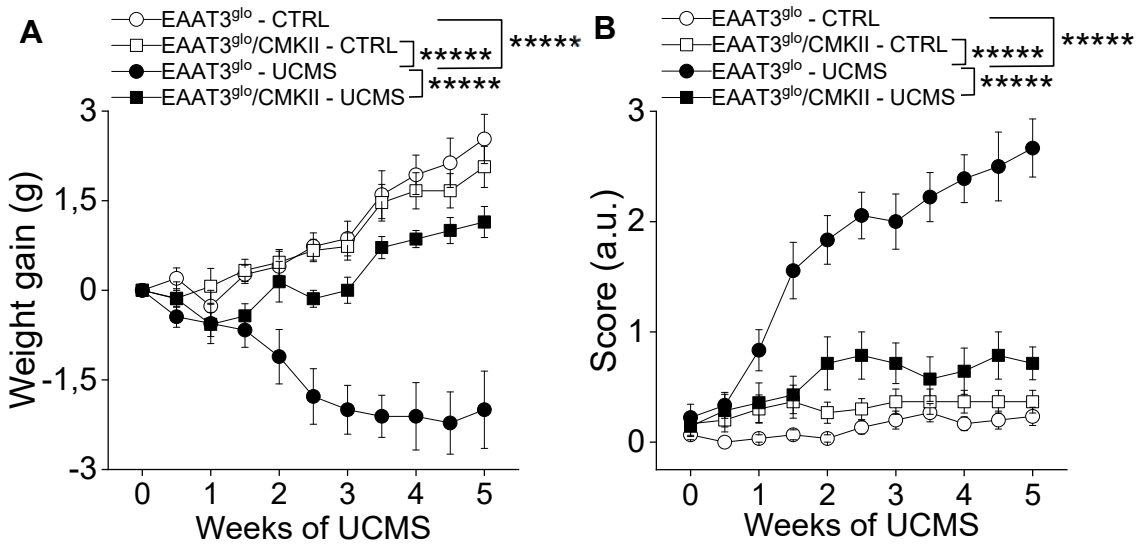

Figure 18. Effects of 5 weeks UCMS on the physical state in EAAT3 overexpression mice. The UCMS had no effect on body weight gain **(A)** and coat state **(B)** in EAAT3^{glo}/CMKII mice. Data are expressed as mean ± SEM; *****p < 0.00001. Control: EAAT3^{glo} n = 15 and EAAT3^{glo}/CMKII n = 14, and UCMS: EAAT3^{glo} n = 9 and EAAT3^{glo}/CMKII n = 7.

3.2.2. Open field test

Significant differences in time spent in center of the open field test were observed between EAAT3^{glo} and EAAT3^{glo}/CMKII mice (Fig. 19B; Two-Way ANOVA, $F(1, 40) = 9.79$, $p < 0.01$). In addition, a main effect of baseline and UCMS conditions was observed (Two-Way ANOVA, $F(1, 40) = 12.91$, $p < 0.001$) and there was interaction between genotype and condition (Two-Way ANOVA, $F(1, 40) = 33.22$, $p < 0.00001$). In baseline conditions EAAT3^{glo}/CMKII mice spent less time in center of the open field test compared to EAAT3^{glo} mice (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo}/CMKII – CTRL, $t = -7.62$, $p < 0.00001$).

EAAT3^{glo} mice challenged to UCMS spent less time in center of the open field arena compared to EAAT3^{glo} mice in baseline conditions (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo} – UCMS, $t = -6.81$, $p < 0.00001$). UCMS had no effect on anxiety levels shown in baseline conditions in EAAT3^{glo}/CMKII mice (*post hoc* Fisher test: EAAT3^{glo}/CMKII – CTRL vs EAAT3^{glo}/CMKII – CTRL, $t = 1.49$, $p = 0.14$).

3.2.3. Sucrose preference and tail suspension tests

Significant differences in sucrose consumption (Fig. 20A; Two-Way ANOVA, $F(1, 41) = 33.66$, $p < 0.00001$) and immobility time (Fig. 20B; Two-Way ANOVA, $F(1, 41) = 7.77$, $p < 0.01$) were observed between baseline and chronic stress conditons. There was a main effect of genotype in the sucrose preference test (Two-Way ANOVA, $F(1, 41) = 4.28$, $p < 0.05$), and in the tail

suspension test (Two-Way ANOVA, $F(1, 41) = 20.54$, $p < 0.0001$). Furthermore, interaction between genotype and condition was observed in the sucrose preference test (Two-Way ANOVA, $F(1, 41) = 12.31$, $p < 0.01$) and in the tail suspension test (Two-Way ANOVA, $F(1, 41) = 13.96$, $p < 0.001$).

EAAT3^{glo} mice subjected to UCMS protocol showed reduced sucrose consumption (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo} – UCMS, $t = -6.91$, $p < 0.00001$) and showed an increase in immobility time (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo} – UCMS, $t = 4.84$, $p < 0.0001$) compared to non-stressed EAAT3^{glo} mice. In baseline conditions, we did not observe differences in sucrose preference between EAAT3^{glo} and EAAT3^{glo}/CMKII (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo}/CMKII – CTRL, $t = -1.21$, $p = 0.23$). Moreover, we found that UCMS did not trigger a decrease in sucrose consumption in EAAT3^{glo}/CMKII mice compared to EAAT3^{glo}/CMKII mice in baseline conditions (*post hoc* Fisher test: EAAT3^{glo}/CMKII – CTRL vs EAAT3^{glo}/CMKII – UCMS, $t = 3.46$, $p = 0.13$), and compared to stressed EAAT3^{glo} mice (*post hoc* Fisher test: EAAT3^{glo} – UCMS vs EAAT3^{glo}/CMKII – UCMS, $t = 3.46$, $p < 0.01$). In addition, UCMS did not induce an increase in immobility time in EAAT3^{glo}/CMKII mice compared to non-stressed EAAT3^{glo}/CMKII mice (*post hoc* Fisher test: EAAT3^{glo}/CMKII – CTRL vs EAAT3^{glo}/CMKII – UCMS, $t = -0.64$, $p = 0.52$), and compared to EAAT3^{glo} mice subjected to UCMS (*post hoc* Fisher test: EAAT3^{glo} – UCMS vs EAAT3^{glo}/CMKII – UCMS, $t = -5.13$, $p < 0.00001$).

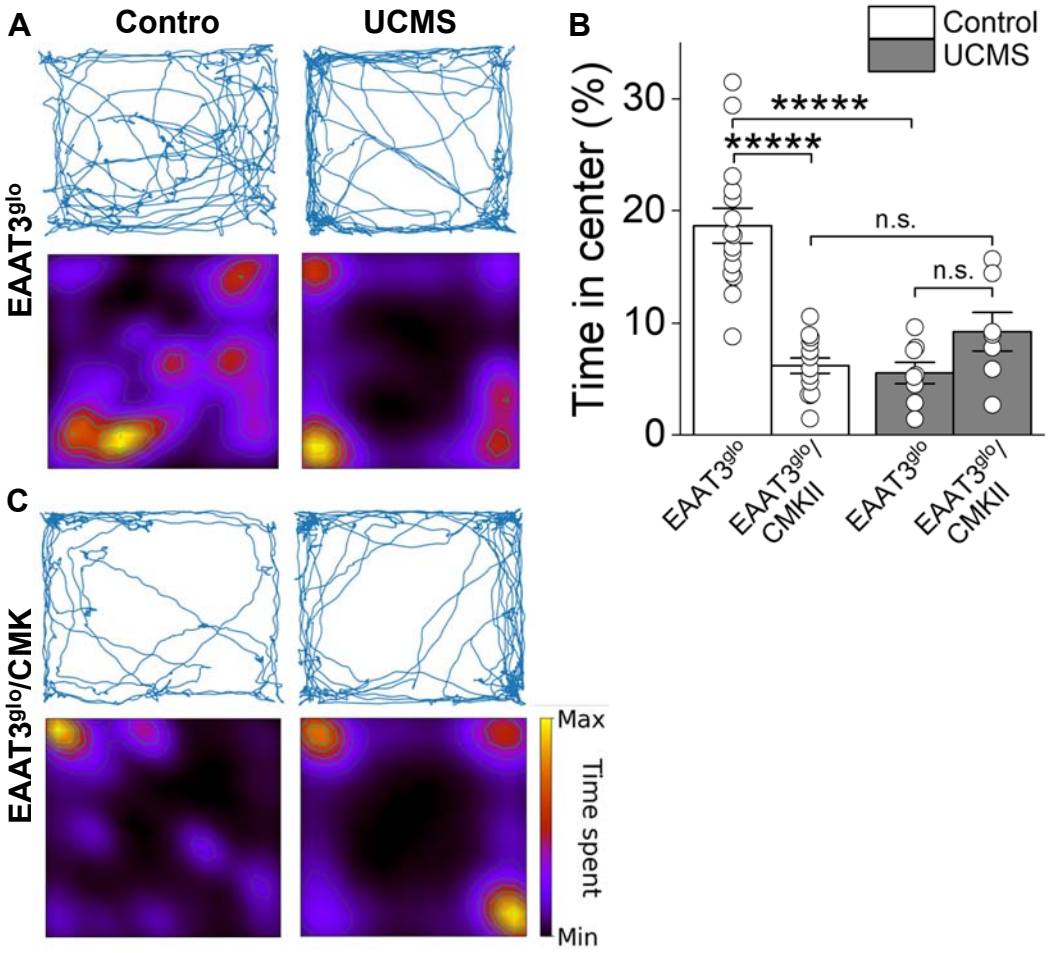

Figure 19. Effects of EAAT3 overexpression driven by CaMKIIα-promoter on anxiety-like behavior in baseline and UCMS conditions. Representative linear (top) and heatmap (bottom) trackings in the open field test for each group **(A** and **C)**; yellow represents increased time spent and dark blue represents minimal time spent over trial. EAAT3 overexpression mice showed significant reduction in time in center in baseline conditions. UCMS triggered anxiety-like behavior in EAAT3^{glo} mice with no effect on EAAT3^{glo}/CMKII mice **(B)**. Data are expressed as mean ± SEM; *****p < 0.00001. Control: EAAT3^{glo} n = 15 and EAAT3^{glo}/CMKII n = 14, and UCMS: EAAT3^{glo} n = 9 and EAAT3^{glo}/CMKII n = 7.

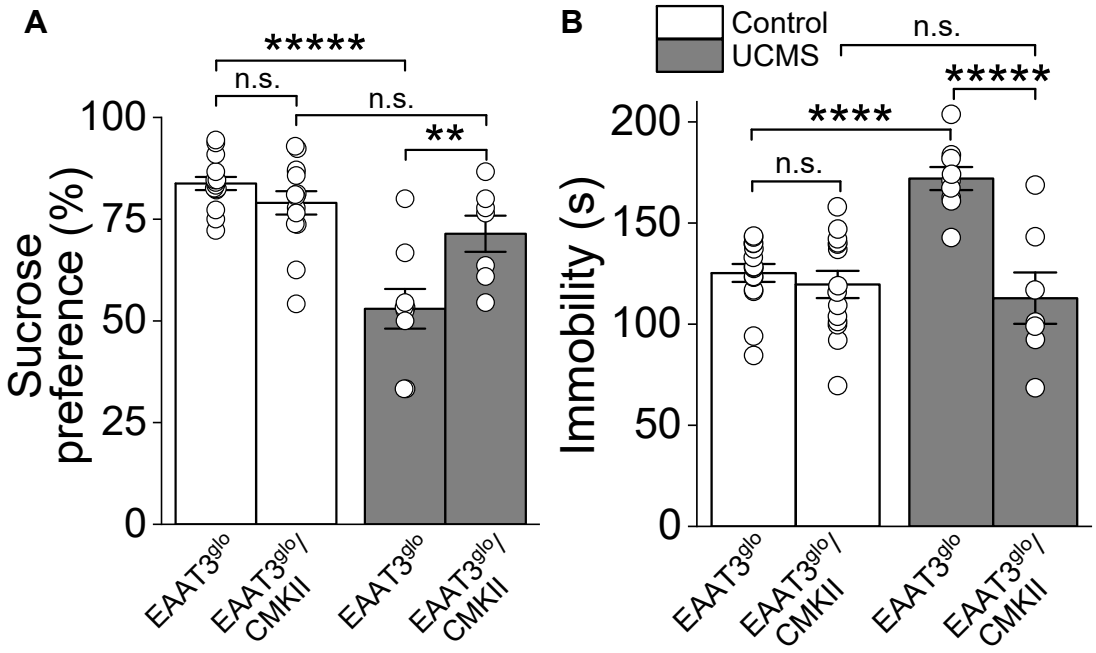

Figure 20. Effects of conditional EAAT3 overexpression driven by CaMKIIα-promoter on depressive-like behavior in baseline and UCMS conditions. EAAT3 overexpression mice challenged to UCMS did neither show anhedonia **(A)** nor behavioral despair **(B)**. Data are expressed as mean ± SEM, **p < 0.01, ****p < 0.0001 and *****p < 0.00001. Control: EAAT3^{glo} n = 15 and EAAT3^{glo}/CMKII n = 14, and UCMS: EAAT3^{glo} n = 9 and EAAT3^{glo}/CMKII n = 7.

3.2.4. Three-chambered social interaction test

We did not find differences in social interaction between EAAT3^{glo} and EAAT3^{glo}/CMKII genotypes (Fig. 21; Two-Way ANOVA, F(1, 41) = 3.43, p = 0.07). There was a main effect of baseline and UCMS conditions (Two-Way ANOVA, F(1, 41) = 15.40, p < 0.001) and there was no interaction between genotype and condition (Two-Way ANOVA, F(1, 41) = 2.02, p = 0.16).

UCMS impaired social interaction in EAAT3^{glo} mice (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo} – UCMS, t = -3.97, p < 0.001). EAAT3^{glo}/CMKII mice subjected to UCMS did not show deficits in social interaction compared to non-stressed EAAT3^{glo}/CMKII mice (*post hoc* Fisher test: EAAT3^{glo}/CMKII – CTRL vs EAAT3^{glo}/CMKII – UCMS, t = -1.69, p = 0.10), and stressed EAAT3^{glo} mice (*post hoc* Fisher test: EAAT3^{glo} – UCMS vs EAAT3^{glo}/CMKII – UCMS, t = 2.03, p < 0.05).

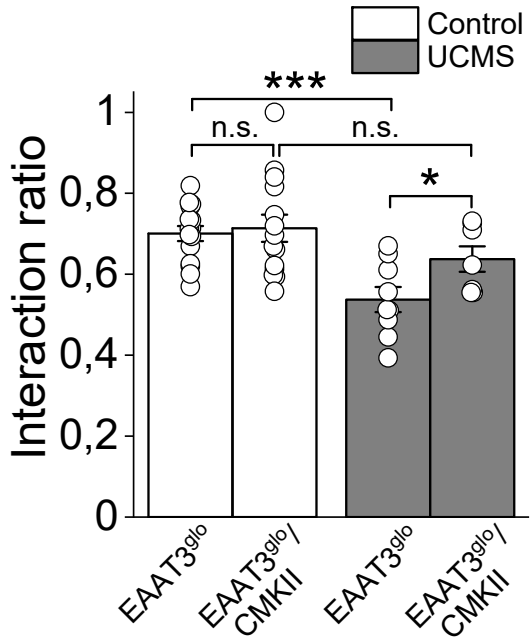

Figure 21. Social interaction in EAAT3^{glo}/CMKII mice at baseline and UCMS conditions. EAAT3 overexpression mice challenged to UCMS did not show déficit in social behavior in three chamber social interaction test. Data are expressed as mean ± SEM; *p < 0.05 and ***p < 0.001. Control: EAAT3^{glo} n = 15 and EAAT3^{glo}/CMKII n = 14, and UCMS: EAAT3^{glo} n = 9 and EAAT3^{glo}/CMKII n = 7.

3.2.5. Depression score

There were a main effect of baseline and chronic stress conditons (Fig. 22; Two-Way ANOVA, F(1, 41) = 52.63 , p <0.00001), and genotype (Two-Way ANOVA, F(1, 41) = 21.63, p < 0.0001) in depression score. Furthermore, interaction between genotype and condition was observed (Two-Way ANOVA, F(1, 41) = 12.31, p < 0.01).

EAAT3^{glo} mice subjected to UCMS protocol showed higher depression score values (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo} – UCMS, t = 9.19, p < 0.00001), reflecting overall depressive-like behavior. In baseline conditions, we did not observe differences between EAAT3^{glo} and EAAT3^{glo}/CMKII (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo}/CMKII – CTRL, t = 0.39, p = 0.69). Stressed EAAT3^{glo}/CMKII mice did not show increase in depression score compared to non-stressed EAAT3^{glo}/CMKII mice (*post hoc* Fisher test: EAAT3^{glo}/CMKII – CTRL vs EAAT3^{glo}/CMKII – UCMS, t = 1.44, p = 0.15), and stressed EAAT3^{glo} mice (*post hoc* Fisher test: EAAT3^{glo} – UCMS vs EAAT3^{glo}/CMKII – UCMS, t = -6.07, p < 0.00001).

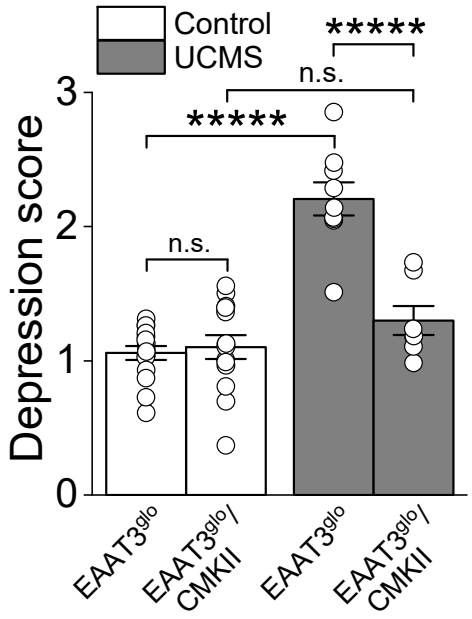

Figure 22. Depression score in EAAT3^{glo}/CMKII mice at baseline and UCMS conditions. EAAT3 overexpression mice challenged to UCMS did not show déficit in social behavior in three chamber social interaction test. Data are expressed as mean ± SEM; *p < 0.05 and ***p < 0.001. Control: EAAT3^{glo} n = 15 and EAAT3^{glo}/CMKII n = 14, and UCMS: EAAT3^{glo} n = 9 and EAAT3^{glo}/CMKII n = 7.

3.2.6. Object location and object recognition memory tasks

To examine the role of EAAT3 overexpression in memory, we evaluated EAAT3^{glo} and EAAT3^{glo}/CMKII mice for long-term OLM and ORM. Significant differences in OLM DI were observed between baseline and chronic stress conditons (Fig. 23 and Fig. 25A; Two-Way ANOVA, $F(1, 17) = 8.29$, $p < 0.05$). Nevertheless, no significant differences in ORM DI were observed between non-stressed and stressed conditions (Fig. 24 and 25B; Two-Way ANOVA, $F(1, 16) = 2.15$, $p = 0.16$). There was a main effect of genotype in OLM DI (Two-Way ANOVA, $F(1, 17) = 4.79$, $p < 0.05$). No genotype effects were observed in ORM DI (Two-Way ANOVA, $F(1, 16) = 1.37$, $p = 0.26$). Furthermore, interaction between genotype and condition was observed in both memory tasks, OLM DI (Two-Way ANOVA, $F(1, 17) = 4.84$, $p < 0.05$) and ORM DI (Two-Way ANOVA, $F(1, 16) = 6.35$, $p < 0.05$).

EAAT3^{glo} mice subjected to UCMS protocol showed impairments in long-term memory, as noted in OLM (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo} – UCMS, $t = -4.56$, $p < 0.001$) and ORM (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo} – UCMS, $t = -3.33$, $p < 0.01$) compared to non-stressed EAAT3^{glo} mice. In baseline conditions, we did not observe differences in OLM DI (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo}/CMKII – CTRL, $t = -0.0099$, $p = 0.99$) and ORM DI (*post hoc* Fisher test: EAAT3^{glo} – CTRL vs EAAT3^{glo}/CMKII – CTRL, $t = -1.04$, $p = 0.31$)

between EAAT3glo and EAAT3glo/CMKII. Moreover, we found that UCMS did not affect long-term memory in EAAT3^{glo}/CMKII mice compared with non-stressed EAAT3^{glo}/CMKII mice, as observed in OLM (*post hoc* Fisher test: EAAT3^{glo}/CMKII – CTRL vs EAAT3^{glo}/CMKII – UCMS, t = -0.41, p = 0.69) and ORM (*post hoc* Fisher test: EAAT3^{glo}/CMKII – CTRL vs EAAT3^{glo}/CMKII – UCMS, t = 0.66, p = 0.52), and compared with stressed EAAT3^{glo} mice in both memory tasks, OLM DI (*post hoc* Fisher test: EAAT3^{glo} – UCMS vs EAAT3^{glo}/CMKII – UCMS, t = 2.95, p < 0.01) and ORM DI (*post hoc* Fisher test: EAAT3^{glo} – UCMS vs EAAT3^{glo}/CMKII – UCMS, t = 2.41, p < 0.05).

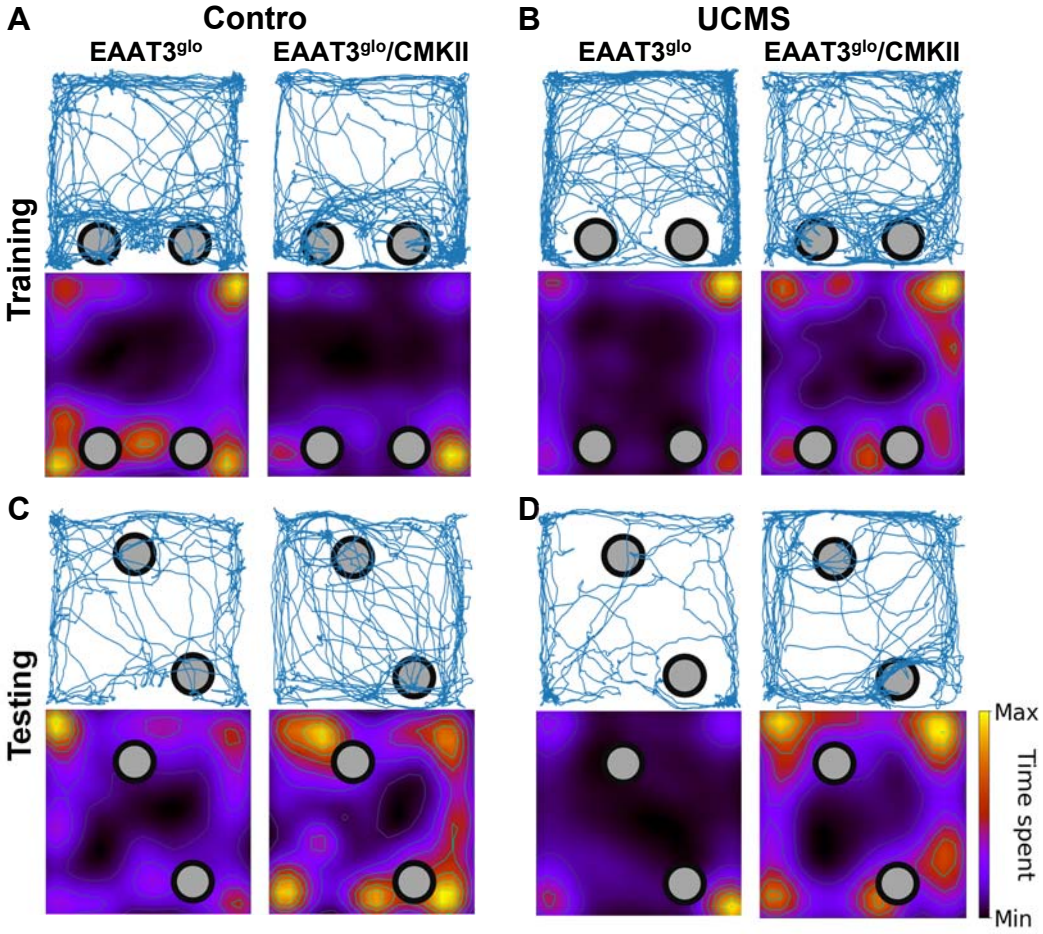

Figure 23. Exploratory behavioral patterns in OLM task in EAAT3^{glo}/CMKII mice at baseline and UCMS conditions. Representative linear (top) and heatmap (bottom) trackings in the OLM training in non-stressed **(A)** and stressed **(B)** mice, and OLM testing at baseline **(C)** and UCMS **(D)** conditions for each genotype; yellow represents increased time spent and dark blue represents minimal time spent over trial.

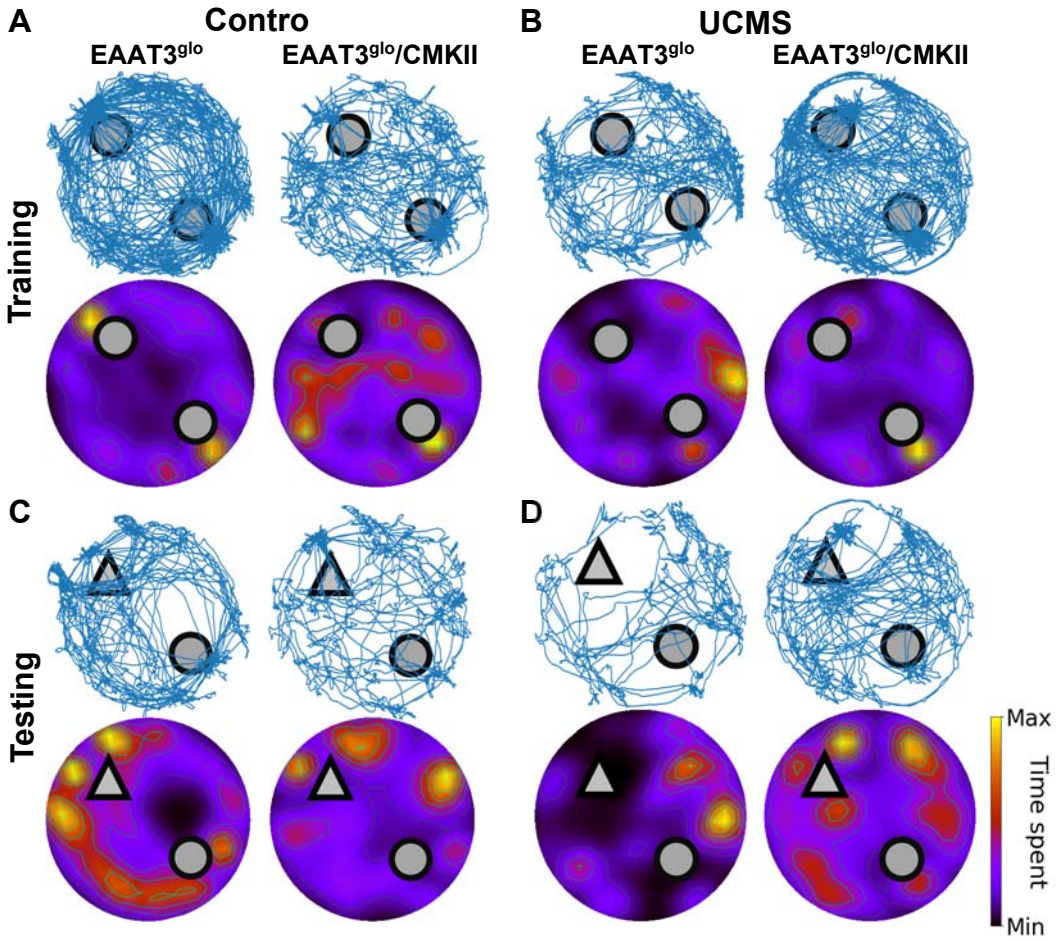

Figure 24. Exploratory behavioral patterns in ORM task in EAAT3^{glo}/CMKII mice at baseline and UCMS conditions. Representative linear (top) and heatmap (bottom) trackings in the ORM training in non-stressed **(A)** and stressed **(B)** mice, and ORM testing at baseline **(C)** and UCMS **(D)** conditions for each genotype; yellow represents increased time spent and dark blue represents minimal time spent over trial.

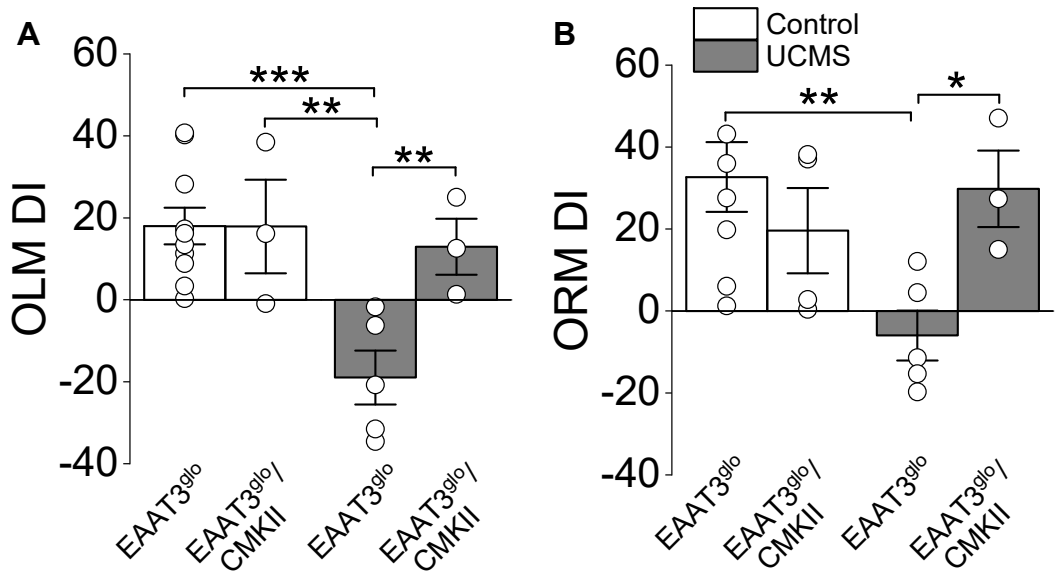

Figure 25. Effects of conditional EAAT3 overexpression on OLM and ORM in baseline and UCMS conditions. Stressed EAAT3^{glo}/CMKII did not show a decrease in OLM DI **(A)** and ORM DI **(B)**. Data are expressed as mean ± SEM; *p < 0.05, **p < 0.01 and ***p < <0.001. OLM, Control: EAAT3^{glo} n = 10 and EAAT3^{glo}/CMKII n = 3, and UCMS: EAAT3^{glo} n = 5 and EAAT3^{glo}/CMKII n = 3. ORM, Control: EAAT3^{glo} n = 8 and EAAT3^{glo}/CMKII n = 4, and UCMS: EAAT3^{glo} n = 5 and EAAT3^{glo}/CMKII n = 3.

3.3. Chronic social defeat stress in WT mice

3.3.1. CF-1 social interaction test

In addition to UCMS, we evaluated the effects of chronic social defeat stress (CSDS) in WT mice at the behavioral level. Behavioral results from social defeat stress are reported by CF-1 social interaction (CF-1 SI) ratio, which is obtained by dividing the sniffing time spent in the interaction zone when the target CF-1 mouse is present by the sniffing time spent in the interaction zone when the target is absent. A CF-1 SI ratio equal to 1, in which equal time is spent in the presence versus absence of a social target, has been used as the threshold for dividing defeated mice into the susceptible and resilient categories (Fig. 26).

We observed differences in the CF-1 SI ratio between control, resilient and susceptible WT mice (Fig. 27; One-Way ANOVA, $F(2, 11) = 5.39$, $p < 0.05$). Post hoc analysis did not reveal significant differences in social behavior between control and resilient (*post hoc* Fisher test: control vs resilient, $t = -0.12$, $p = 0.91$), although we found differences between control and susceptible (*post*

hoc Fisher test: control vs susceptible, t = -3.06, p < 0.05), and resilient and susceptible WT mice (*post hoc* Fisher test: resilient vs susceptible, t = -2.67, p < 0.05).

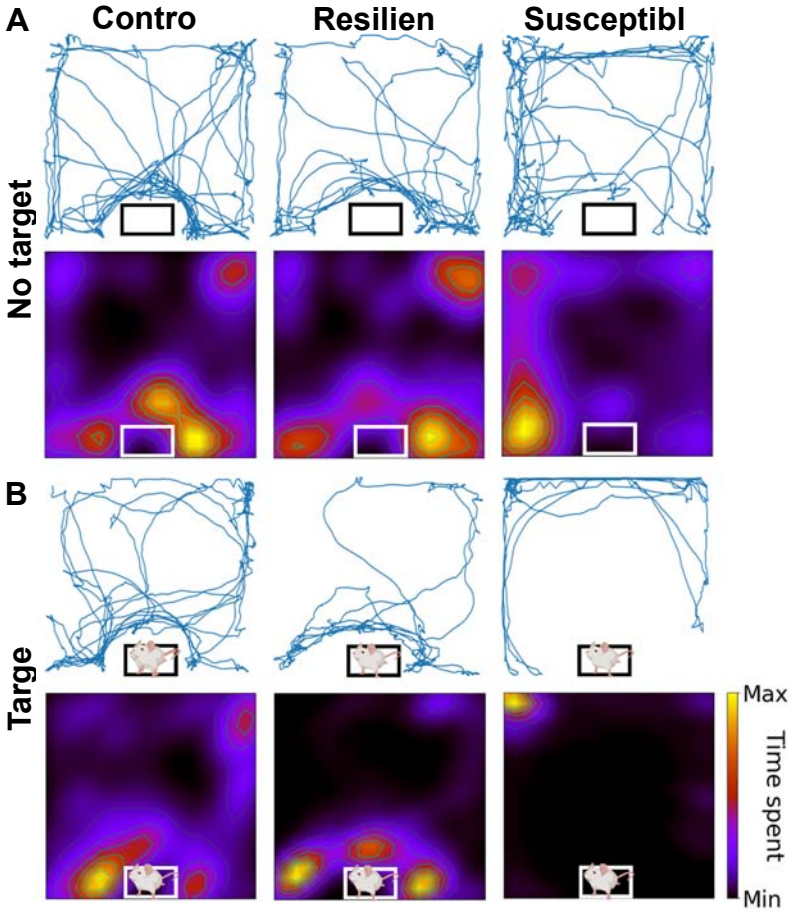

Figure 26. Effects of CSDS on social interaction to CF-1 mouse. Representative linear (top) and heatmap (bottom) trackings of social interaction data for control, susceptible and resilient mice in the absence of a target CF-1 mouse **(A)**, and in the presence of a target CF-1 mouse **(B)**; yellow represents increased time spent and dark blue represents minimal time spent over trial.

3.3.2. Body weight gain

We found significant differences in body weight gain between control, resilient and susceptible WT mice (Fig. 28; Two-Way Repeated Measures ANOVA, F(12.2, 61.3) = 3.39, p < 0.001). CSDS significantly reduced the body weight gain in resilient and susceptible mice from day 3 (*post hoc* Fisher test: Control vs Resilient, t = 7.18, p < 0.0001; Control vs Susceptible, t = 14.87, p < 0.00001). From day 11 significant differences in weight gain between resilient and susceptible mice were observed (*post hoc* Fisher test: Control vs esilient, t = 7.29, p < 0.0001). These changes

were maintained over time.

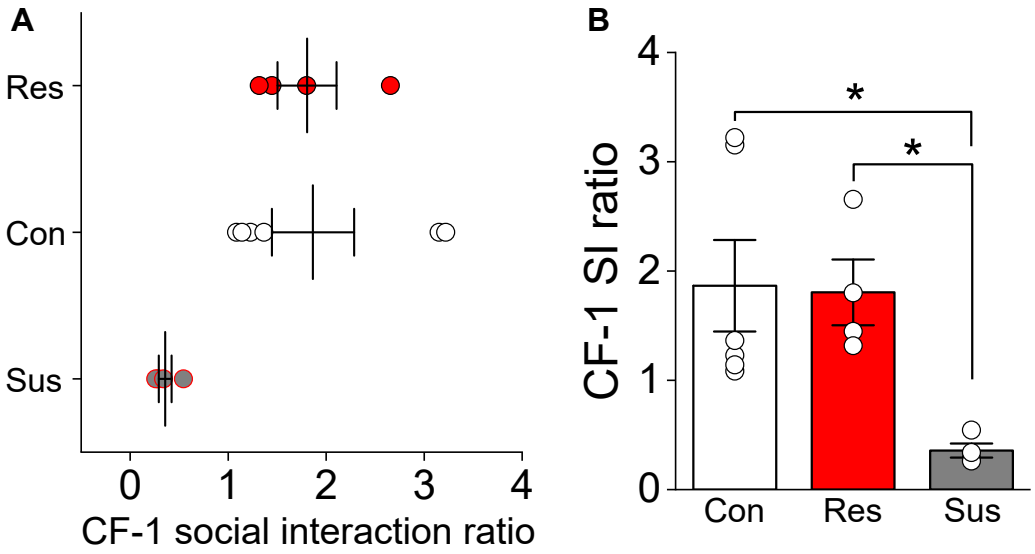

Figure 27. CSDS induced avoidance behavior in susceptible WT mice. Repeated social defeat stress resulted in a spectrum of social behavior, divided between susceptible and resilient phenotypes as a function of their SI ratio score **(A)**. Susceptible WT mice showed social avoidance **(B)**. Data are expressed as mean ± SEM; *p < 0.05. Control (Con) n = 6, resilient (Res) n = 4 and susceptible (Sus) n = 4.

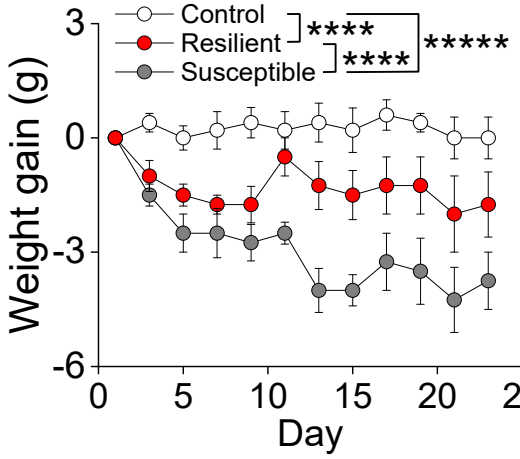

Figure 28. Effects of 21 days of social defeat on body weight gain. CSDS significantly disrupted the body weight gain in susceptible and resilient WT mice. Data are expressed as mean ± SEM; ****p < 0.0001 and *****p < 0.00001. Control n = 6, resilient n = 4 and susceptible n = 4.

3.3.3. Open field test

We did not find significant differences between control, resilient and susceptible WT mice in time spent in the center of the open field arena (Fig. 29B; One-Way ANOVA, $F(2, 11) = 3.03$, $p = 0.80$), entries to center (Fig. 29C; One-Way ANOVA, $F(2, 11) = 2.42$, $p = 0.13$) and horizontal activity (Fig. 29D; One-Way ANOVA, $F(2, 11) = 0.81$, $p = 0.47$).

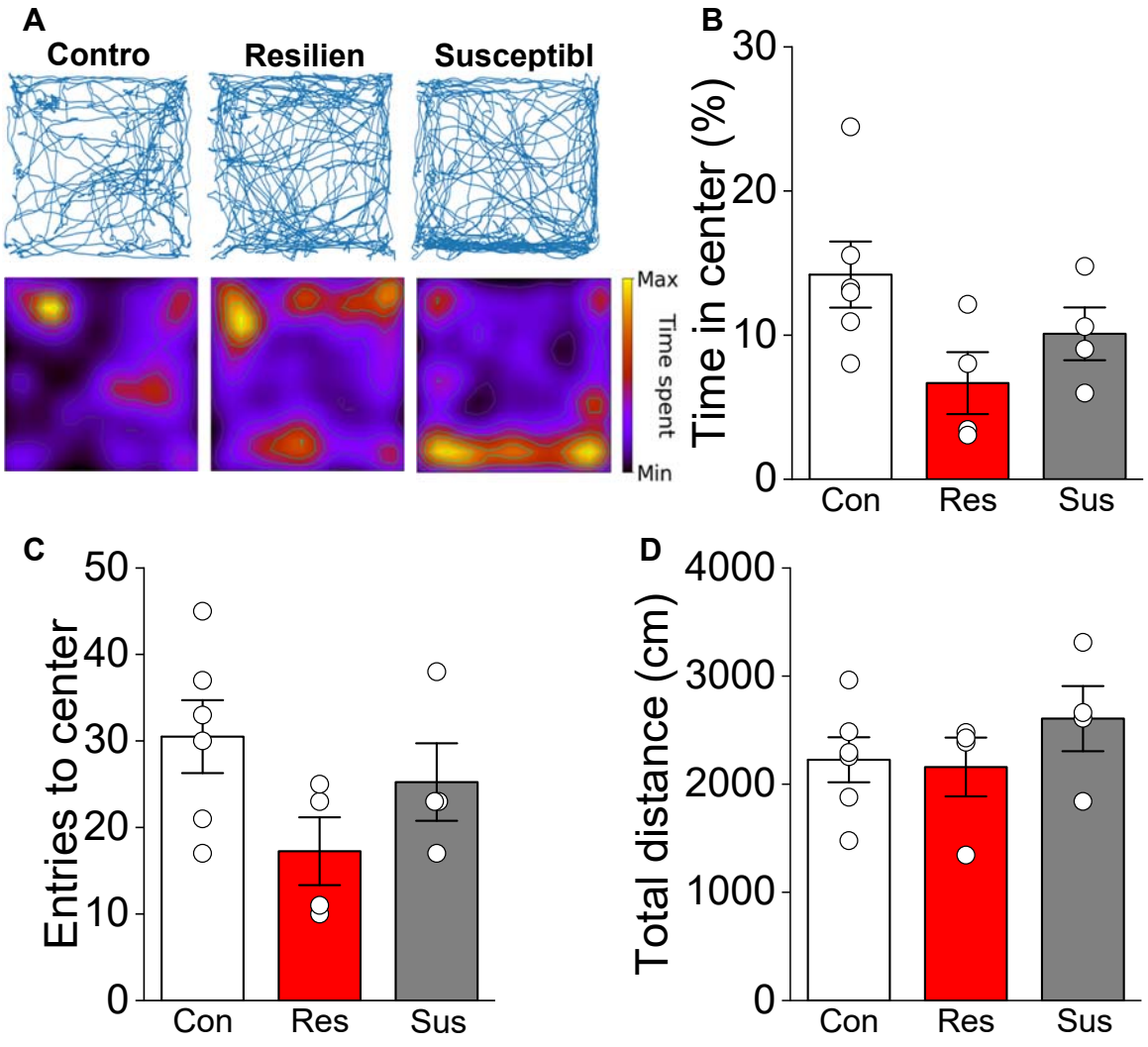

Figure 29. Effects of CSDS on anxiety-like behavior and locomotory patterns in WT mice. Representative linear (top) and heatmap (bottom) trackings in the open field test for each group **(A)**; yellow represents increased time spent and dark blue represents minimal time spent over trial. No significant differences between control, resilient and susceptible WT mice were seen for time spent in center **(B)**, frequency to visit the center of arena **(C)** and total distance **(D)**. Data are expressed as mean ± SEM. Control (Con) n = 6, resilient (Res) n = 4 and susceptible (Sus) n = 4.

3.3.4. Sucrose preference and tail suspension tests

Repeated social defeat did not affect sucrose consumption (Fig. 30A; One-Way ANOVA, $F(2, 11)$

= 1.26, p = 0.32). At the end of the CSDS, differences between control, resilient and susceptible WT mice in the tail suspension test were observed (Fig. 30B; One-Way ANOVA, F(2, 11) = 5.88, p < 0.05). We found that susceptible mice showed a tendency to increase immobility time compared to control group (*post hoc* Fisher test: Control vs Susceptible, t = 2.17, p = 0.05), and a significant increase in immobility time compared to resilient mice (*post hoc* Fisher test: Resilient vs Susceptible, t = 3.41, p < 0.01). No significant differences between control and susceptible WT mice were observed (*post hoc* Fisher test: Control vs Resilient, t = -1.57, p = 0.15).

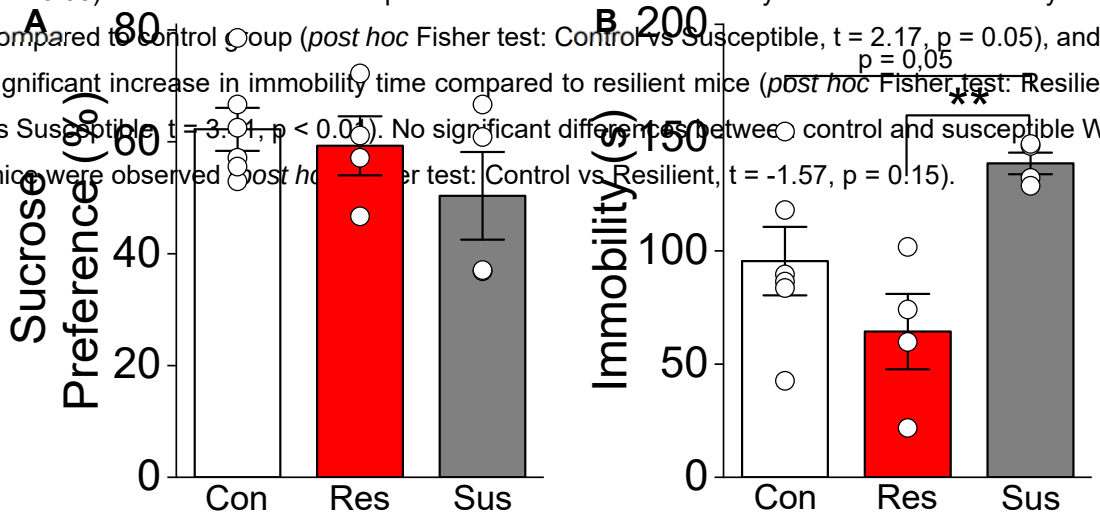

Figure 30. Effects of CSDS on depressive-like behavior in WT mice. No significant differences between control, resilient and susceptible WT mice were seen for sucrose consumption **(A)**. WT susceptible mice showed more immobility time compared to control and resiliente mice **(B)**. Data are expressed as mean ± SEM; **p < 0.01. Control (Con) n = 6, resilient (Res) n = 4 and susceptible (Sus) n = 4.

3.3.5. Depression score

To get an overview of depressive-like behavior in WT mice subjected to social defeat, we used the depression score to include the results of CF-1 social interaction test, sucrose preference test, and tail suspension test.

Significant diferences in depression score between control, resilient and susceptible WT mice were observed (Fig. 31; One-Way ANOVA, F(2, 11) = 7.71, p < 0.01). Susceptible mice showed higher depression rates compared to control and resilient mice (*post hoc* Fisher test: control vs susceptible, t = 3.12, p < 0.01; resilient vs susceptible, t = 3.70, p < 0.01). No significant differences were observed in depression score values between control and resilient groups (*post hoc* Fisher test: control vs resilient, t = -0.93, p = 0.37).

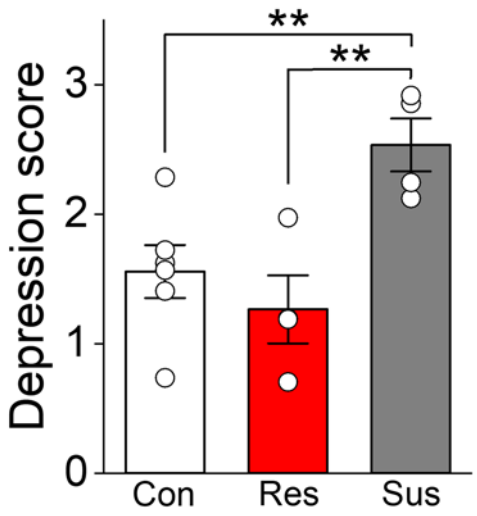

Figure 31. Effects of CSDS on global depression score in WT mice. WT mice susceptible to social defeat showed higher depression score values. Data are expressed as mean ± SEM; **p < 0.01. Control (Con) n = 6, resilient (Res) n = 4 and susceptible (Sus) n = 4.

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4. Discussion

4.1. Unpredictable chronic mild stress

Chronic stress dysregulates the homeostasis of the organisms and is considered among the causes that contributes to the etiology of MDD (Kendler et al., 1999). In order to study the mechanisms underlying depression we used the UCMS model, a well-stablished animal model of depression (Nollet et al., 2013; Burstein and Doron, 2018). In our laboratory, we have successfully established the UCMS paradigm in mice. After five weeks of daily subjecting WT mice to UCMS, individuals showed deficits in weight gain, deterioration of coat state, and anxiety- and depressive-like behaviors.

A depression diagnosis comprises a multiple comprehensive screening based on clinician-rated scales and self-report questionnaires, which assess daily moods, behaviors, and lifestyle habits (Uher et al., 2012; American Psychiatric Association, 2013). Likewise, we calculated a depression score to integrate the behavioral tests battery related to depression as an endpoint to evaluate depressive-like behaviors in mice. Therefore, stressed WT mice showed a higher depression score compared to non-stressed WT mice, indicating UCMS-triggered global depressive-like behavior. Together, these changes are referred to as a depressive behavioral profile.

In rodents, the frequency and pattern of self-grooming behavior is very sensitive to stress (Kalueff and Tuohimaa, 2004). Grooming behavior is relevant to maintain the physiological homeostasis, comfort and appearance of the mouse (Kalueff et al., 2016). Deterioration of coat state observed in chronically stressed mice can be related to disturbances in the pattern and frequency of grooming. The body parts most affected by stress were the head, the neck, the back and the abdomen. In stressed WT mice we observed that the maximum value of coat state does not exceed 4. Impairment in self-grooming in stressed mice would be correlated to poor personal hygiene and decreased self-care behaviors shown by depressed patients.

The DSM-5 reports gain or loss of body weight as a symptom of depression. Whereas there are studies that report an increase in body weight (Konttinen et al., 2019; Sahle et al., 2019), other studies have indicated a tendency to lose body weight in MDD patients (Chaitoff et al., 2019). Body weight gain or loss could be due to metabolism, body mass index or eating habits of each individual (Geliebter and Aversa, 2003). Furthermore, higher insulin concentrations in people with

higher body mass index (Dallman, 2010) and individual differences in ghrelin level and response (Sominsky and Spencer, 2014) may contribute to the variability in stress-induced body weight alterations. In animal models, chronic stress induces a decrease in body weight related to anhedonia in different species of rodents (Willner et al., 1996). On the contrary, Swiss and BALB/c mice challenged to UCMS showed a significant body weight gain (Yalcin et al., 2008). Nevertheless, other studies have not found significant differences between control and UCMS mice (Surget et al., 2008). Because all individually stressed mice do not cope with stress in the same way, it is not surprising to find differences in body weight changes between different animal models of stress-induced depression, similar as observed in humans. In addition, metabolism and genetic background of animals as well as duration, scheduling and type of stressors applied could influence the variability in stress-induced body weight changes. According to the changes in body weight observed in depressed humans and animal models of depression, our results robustly showed weight loss in WT mice subjected to UCMS. Stress-triggered homeostatic disruption involves the HPA axis and the sympathetic nervous system. In acute stress, the HPA response is driven primarily by CRH released from hypothalamic PVN neurons and CRH-induced ACTH reléase from the anterior pituitary (Myers et al., 2012). In acute stress, by inhibiting the appetite-stimulating neuropeptide Y (NPY) in arcuate nucleus in the hypothalamus, CRH inhibits feeding (Heinrichs and Richard, 1999; Currie, 2003), thereby prioritizing energetic resources to respond quickly to stressor. ACTH-stimulated GCs secretion from adrenal cortex inhibits CRH release, which increases NPY release in the hypothalamus and restores the homeostasis (Pralong et al., 1993). On the other hand, studies postulate weight gain under conditions of chronic stress-induced hypercortisolemia (Pecoraro et al., 2004), presumably as a compensatory response to the deleterious effects of stress. Controversially, as mentioned above, both human and animal studies have shown bidirectional results regarding the changes in body weight in chronic stress conditions. Weight loss in stressed WT mice could be related to decreased motivation to eat their regular food. Since studies propose that chronic stress generates an increase in palatable food, in future research would be suggestive to evaluate whether mice subjected to chronic stress prefer to consume regular or palatable food.

Although many of the symptoms of MDD, such as suicidal ideation and depressed mood cannot be replicated in animal models, UCMS animals show a long-lasting maladaptative phenotype related to symptoms of human depression, such as anhedonia, impairments in social interaction, learning deficits, behavioral despair, and sleep disturbances (Nollet et al., 2013; Willner, 2016). We used anhedonia, behavioral despair and sociability as behavioral endpoint to assess the effects of UCMS. In line with this focus, we found significant differences between non-stressed and stressed WT mice in the sucrose preference test, thereby resulting in UCMS-induced

anhedonia. In rodents, the hedonic gustative pathway integrates information from sensory areas and sends inputs to the NAc shell (shNAc) and the VTA (Scheggi et al., 2018). Furthermore, consumption of sucrose, considered as a palatable food for rodents, increases DA levels in the NAc in a concentration-dependent manner (Hajnal et al., 2004). Consequently, UCMS mice could be impaired in the reward system. In the tail suspension test, mice challenged to UCMS showed longer immobility time. The tail suspension test is not only a well-validated test for the evaluation of antidepressant efficacy of drugs, but also is used to assess the effects of environmental, neurobiological and genetic manipulations (Liu and Gershenfeld, 2001; Cryan et al., 2005; Castagné et al., 2011).

Both depressed humans and animal models of depression exhibit impairment in social interaction (D'Aquila et al., 1994; Kupferberg et al., 2016; Van Boxelaere et al., 2017). In addition to the relevance for emotional well-being, healthy state and playing a crucial role in development (Bicks et al., 2020), social interaction is also considered a rewarding stimulus in mammals (Trezza et al., 2011). Our results indicate that 5-week UCMS schedule are sufficient to generate deficits in social behavior in WT mice. The interaction with another mouse of the same strain and the same sex is a stimulus previously experienced by the evaluated mouse, since before starting the UCMS protocol the mice were group-housed of 3-5 littermates. Mice subjected to UCMS were singly housing as an additional stressor. Unlike the three-chambered social interaction test, the sucrose preference test was the first exposure to a sweet rewarding stimulus for all mice. That is, the type of anhedonia displayed by stressed mice was due to a rewarding novelty and not to pleasure previously experienced. Therefore, anhedonia can be defined under different behavioral domains and neurobiological constructs. The ability to anticipate or predict expected rewards may be affected in the social interaction déficit. The ventral tegmental area (VTA) and Amy are the main brain areas responsible for anticipation and motivation to carry out the goal-directed activity, which are connected to the NAc, the ventral pallidum and the intracortical network, mediating the perception of pleasure, reward value and the perception of pleasure (Der-Avakian and Markou, 2012). The mPFC, which is innervated by dopaminergic inputs from the VTA and GABAergic from the NAc, is involved in the decision to participate in a goal-directed activity to obtain the pleasant stimulus (Der-Avakian and Markou, 2012). Since UCMS individuals showed deficits in anhedonia and social interaction, many areas involving the circuit related to reward processing could be affected by chronic stress. The reciprocal connections of PFC with NAc, VTA, Amy and Hpc could play an important role in the regulation of the behavioral response to rewards, besides glutamatergic signaling could be mediating the activity of this circuit (Floresco et al., 2001; Bagot et al., 2015; Bossong et al., 2018).

Using OLM and ORM tasks we evaluated the consequences of UCMS on long-term episodic memory in mice. OLM and ORM tests rely strongly on visual recognition memory, rodent innate exploratory behavior and spontaneous preference for new objects and environments (Ennaceur and Delacour, 1988; Vogel-Ciernia and Wood, 2014). A number of line of evidence suggest that chronic stress in rodents is able to impair the short- and long-term memory of object recognition and location (Elizalde et al., 2008; Hattiangady et al., 2014; Román-Albasini et al., 2020). Thereby, according to previous reported works, we have found that, compared to unstressed WT mice, UCMS-mice showed impairment in long-term memory.

Catecholamines and GCs hormones released from the adrenals during stressful events influence the functioning and connectivity of the Hpc, PFC, Amy and Str (Williams and Clayton, 2004; Herman et al., 2005; Myers et al., 2014). At the cellular level, GCs may act on different time scales. Intracellular GC receptors (GRs) and mineralocorticoid receptors (MRs) mediate genomic actions whereas membrane-bound receptors allow rapid, non-genomic GC actions (Joëls et al., 2012). The differential effects of cortisol (corticosterone in rodents) may be due to the difference in affinity between the two types of receptors, MRs are activated by low levels of GCs, while the lower-affinity GRs are fully activated only under conditions of high cortisol levels (Joëls, 2006). The effect of stress on enhancing or impairment of cognitive functions varies according to stressor, time and frequency of exposure. Acute stress is thought to enhance working memory to optimize coping with the stressful event but does not improve retrieval (Schwabe, 2017). A proposed mechanism is based on the evidence that corticosterone may facilitate hippocampal long-term potentiation (LTP), when high levels of the corticosterone and high-frequency stimulation coincide in time, and non-genomic actions predominate (Wiegert et al., 2006). Nonetheless, genomic GC actions were found to inhibit hippocampal LTP (Kim and Diamond, 2002). Moreover, low levels of corticosterone (sufficient to activate a portion of the MRs) have been associated with little calcium influx and efficient LTP, whereas stress slowly impairs LTP induction. This dual action observed with low versus high levels of corticosterone was related to the involvement of the MRs and the GRs, respectively (Joëls, 2006).

The Hpc is one of the main targets of GCs and plays a key role in working and spatial memory, relating emotional aspects of memory with Amy and the hypothalamus, and linking cognitive performance through the connectivity with PFC (Maggio and Segal, 2019). In animal studies, prolonged elevations of corticosterone circulating can potentiate excitotoxicity of hippocampal pyramidal neurons and chronic administration of high doses of corticosterone leads to hippocampal neuronal loss (Sapolsky et al., 1985; Popoli et al., 2012). In addition, impaired induction of LTP in the hippocampal CA1 area elicited by stress and high doses of corticosterone (Shors et al., 1989; Diamond et al., 1992; Alfarez et al., 2002) has been strongly associated with

deficits in spatial and object recognition memory (Radecki et al., 2005; Park et al., 2015). High levels of corticosterone in chronic stress conditions activate intracellular GRs (Park et al., 2015), consequently leading to an increase in glutamate release and dysfunctional clearance (Howland and Wang, 2008; Popoli et al., 2012). Therefore, the activation of extrasynaptically localized NMDA receptors GluN2B subunits through glutamate spillover may facilitate the induction of forms of synaptic plasticity and excitotoxicity mechanisms that lead to disrupted memory performance (Howland and Wang, 2008; Osborne et al., 2015). Moreover, stress can deregulate ionotropic glutamate receptor function and expression levels (Murrough et al., 2017). In this context, we found an increase in the levels of AMPA receptors GluA1 subunit and NMDA receptors GluN2A and GluN2B subunits in the vHpc in WT mice subjected to UCMS. Stress-triggered upregulation of NMDA receptors may be inducing a long-lasting increase in basal synaptic transmission of Hpc by activating NMDA receptors, leading to an increase in the contribution of calcium-permeable AMPA receptors GluA1 subunit to synaptic transmission and LTP impairment, as observed in a neurodegeneration study (Diogenes et al., 2012). Hence, the increased expression levels of AMPA receptors GluA1 subunit and NMDA receptors GluN2A and GluN2B subunits may be impairing LTP induction as a mechanism underlying the deficits observed in OLM and ORM tests in chronically stressed WT mice. However, further studies are required to reveal the physiological mechanism related to increased AMPA and NMDA receptors expression levels in the vHpc of stressed WT mice reported in this study.

Unlike WT and EAAT3^{glo} mice subjected to UCMS, stressed EAAT3^{glo}/CMKII mice did neither show deficits in weight gain nor deterioration of coat state. According to previous studies, EAAT3^{glo}/CMKII mice showed anxiety-like behaviors in basal conditions (Delgado-Acevedo et al., 2019). Exposure to UCMS did not change the level of behavioral anxiety in EAAT3^{glo}/CMKII mice. Surprisingly, conditional EAAT3 overexpression in principal forebrain neurons protected the mice from the negative effects of chronic stress, as observed in the battery of behavioral tests related to depression, and also verified by integrating the data of sucrose preference test, tail suspensión test and three-chambered social interaction test of each individual in the depression score analysis. Interestingly, we found that forebrain EAAT3-overexpressing mice subjected to UCMS did not show deficits in object recognition and location memory. These results suggest a possible neuroprotective mechanism provided by the increase EAAT3 expression against dysregulation in the glutamatergic system and maladaptative behaviors observed in UCMS EAAT3^{glo} and WT mice. Altogether, our results reveal that EAAT3 overexpression in the forebrain of mice is closely linked to a chronic stress resilient phenotype.

One of the mechanisms that could be involved in the dysregulation of glutamatergic signaling in mood disorders is the increase in glutamate spillover, which occurs when glutamate diffuses from

the synapse and activates extrasynaptic receptors or different active zones in the same synapse, enhancing excitatory transmission that may eventually result in neurotoxic cell death (Tzingounis and Wadiche, 2007; Hardingham and Bading, 2010). Studies on chronic stress in rats have shown that stress-induced extracellular glutamate accumulation, accompanied by enhanced GluN2B-mediated NMDA currents and extrasynaptic responses, underlie a depressive-like phenotype (Li et al., 2018). Since the perisynaptic location in postsynaptic neurons, and the role in controlling glutamate spillover (Underhill et al., 2014), the conditional EAAT3 overexpression in the forebrain of mice may be playing a protective role from the negative effects of chronic stress. To study the therapeutic potential in mood disorders, future studies should be focused on finding strategies to increase the levels of EAAT3 after subjecting WT mice to chronic stress, to evaluate if this approach is able to reversing stress-induced changes.

4.2. Changes in glutamate transporters and receptors in WT mice

In evaluating at the protein level in the brain areas involved in the circuit responsible for encoding the response to rewarding stimuli, no significant differences were observed in the levels of EAAT1, EAAT2 and EAAT3 in the mPFC, Str, NAc and Hpc. Although studies report decreased expression levels of glutamate transporters (Zink et al., 2010; Zhang et al., 2013), the differences with our results might be due to differences in the applied depression model and the rodent species used. Nevertheless, these studies shed light on a possible role of glutamate transporters in the pathophysiology of depression. Moreover, in stressed WT mice an upward trend in EAAT1 expression levels in the Hpc was observed ($p = 0.05$). Despite the high variability in EAAT1 protein levels within the UCMS mice group, perhaps a possible increase in hipocampal EAAT1, a glutamate glial transporter, would be increasing glutamate recycling, thereby contributing to the increase in glutamatergic activity observed in patients and animal models of depression. Furthermore, it would be interesting to evaluate the glutamate transport of EAATs in mice under chronic stress conditions.

In relation to ionotropic glutamate receptors expression level, we found significant stress-induced upregulation of NMDA receptors GluN2A and GluN2B subunits, and AMPA receptors GluA1 subunits in the vHpc of WT mice. In the mPFC, Str and NAc we found no significant stress-induced changes in GluN2A and GluN2B subunits, and GluA1 subunits. In addition, we also did not observe changes in the GluA2 expression levels in the mPFC, Str, NAc and vHpc in WT mice subjected to UCMS. In the determinations of protein expression levels, a high variability was observed between mice within the same group. This raises the question of whether the noted variability is due to the low total number of mice used in this study or if this is one of the limitations in the stress model, or also to the individual susceptibility differences of each individual.

Research has shown that GluN2A and GluN2B subunits may both be involved in the induction of LTP. Conversely, just GluN2A subunits could participate in the induction of long-term depression (LTD), while GluN2B subunits appear to not be involved in LTD induction (Bartlett et al., 2007). On the other hand, synaptic insertion of AMPA receptors GluA1 subunits is known to play an important role in mediating the increase in synaptic strength during LTP (Hayashi, 2000; Malinow and Malenka, 2002). Hippocampal LTP has been associated with learning and memory (Hölscher, 1999; Radecki et al., 2005; Whitlock et al., 2006). Nevertheless, other studies question whether LTP can be considered a neural mechanism underlying memory and learning.(Shors and Matzel, 1997; Stevens, 1998; Abraham et al., 2019). Moreover, changes in excitability within neural circuits related to hippocampal LTP might also have undesirable or adverse effects, as observed in the case of susceptibility and epileptiform activity in rats (Chang et al., 2007b). Therefore, LTP-like synaptic plasticity in the Hpc could contribute to increasing glutamate neurotransmission subserving to chronic stress-triggered depressive-like behaviors in mice. Nevertheless, as mentioned above, the results observed in chronically stressed WT mice regarding the excessive increase in the expression levels of calcium-permeable GluA1-containing AMPA receptors probably induced by upregulation of NMDA receptors GluN2A and GluN2B subunits may be impairing LTP induction. Therefore, in future studies it would be of considerable interest to evaluate at the electrophysiological level the consequences of UCMS in hippocampal synaptic plasticity.

Despite the levels of glutamate transporters do not decrease in the UCMS model in WT mice, this does not exclude the possibility of an increase in the levels of one of the glutamate transporters may prevent the deleterious effects of chronic stress in depressive-like behaviors. Our results are in agreement with this hypothesis, by showing that EAAT3 CaMKIIα-promoter driven overexpression mice subjected to UCMS did not show depressive-like behaviors, and no impairment in sociability. To date, few animal models have studied the impact of manipulating the levels of glutamate transporters *in vivo* on mood disorders (Mineur et al., 2007; Cui et al., 2014; John et al., 2015).

4.3. Chronic social defeat stress in WT mice

Unlike UCMS, not all mice subjected to social defeat paradigm are susceptible to stress. A population appears to be resilient to CSDS according to the resistance to defeat-induced avoidance. Furthermore, we observed these differences in the evaluation of body weight gain, in which susceptible WT mice show more dramatic weight loss than resilient WT mice. Nonetheless, we did not find significant differences between susceptible and resilient groups when they were evaluated in anxiety-like behaviors in the open field test, and in preference to sucrose. In this model, to evaluate coat state does not make sense, since the coat is damaged by daily agonistic

encounters between C57BL/6J mice subjected to social defeat and aggressive CF-1 mice. Moreover, in the tail suspensión test, susceptible WT mice spent significantly more immobility time than control and resilient WT mice, and no significant differences were observed between control and resilient mice. Including the data from the CF-1 social interaction test, sucrose preference and the tail suspension test, we significantly found a higher depression score in susceptible WT mice compared to control and resilient groups, in which no significant differences were observed. Our results indicate that social avoidance against an unknown aggressive mouse as the sole criterion for segregating resilience and susceptibility to social defeat seems to be insufficient or incomplete. Besides, to evaluate the sociability of defeated mice against a non-aggressive conspecific mouse, the three-chambered social interaction test could be included in the battery of behavioral tests, thereby evaluating sociability as a hedonic construct rather than social approach-avoidance against an aggressive mouse.

4.4. Conclusion

The type of stressors used in the two animal models of depression resulted in differences in anxiety- and depressive-like behaviors in WT mice. Perhaps social defeat only concern hopelessness and the reward domains associated with sociability and not the hedonic circuit associated with sucrose consumption. On the other hand, despite the fact that a different aggressive CF-1 mouse is used each day in CSDS, the type of psychosocial stressor is the same during the 21 days of social defeat. Thus, the CSDS could be considered as a behavioral paradigm of homotypic stress. Different is the case of UCMS, in which stressors are applied in a random and unpredictable fashion.Exposure to homotypic stress can result in habituation of the HPA axis response, characterized by a decreasing GC response over time (Herman et al., 2016), probably resulting in lower detrimental consequences than UCMS. This evidence could be one of the factors influencing the differences in the behavioral outcomes that we observed between UCMS and CSDS. Although UCMS was more robust in generating depressive-like behavior in WT mice, CSDS confers the advantage of obtaining a resilient population and studying the mechanisms associated with stress resilience.

UCMS-induced increase in protein expression levels of EAAT1, NMDA receptors GluN2A and GluN2B subunits, and AMPA receptors GluA1 subunits in the Hpc in WT mice were associated with anxiety-like behavior, anhedonia, behavioral despair, and deficits in sociability and long-term memory. Despite research proposes to NAc as a central area of information processing of cortical-limbic circuit in motivated behaviors (Goto and Grace, 2005, 2008), the Hpc could play a fundamental role in goal-directed behaviors and motivation, which are affected in mood disorders. Based on our results, we suggest that stress-induced upregulation of ionotropic glutamate

receptors in the vHpc may be related to negative bias in memory and learning in depression (Zhang et al., 2019).

Mice with EAAT3 overexpression driven by CaMKIIα-promoter challenged to UCMS did neither show depressive-like behaviors nor impairment in sociability. Additionally, EAAT3-overexpressing mice subjected to UCMS did not show deficits in object recognition and location memory. These results suggest that EAAT3 overexpression in the forebrain may be linked to a resilient phenotype to UCMS.

Pursuing strategies to rescue or reverse stress-induced alterations in the glutamatergic system could quickly and effectively alleviate not only the main symptoms of depression, but also deficits in cognitive performance.

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