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Antidepressant action of ketamine in two genetic models of impaired glutamate and melatonin function

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A mi Familia

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Abbreviations

5-HT	5-hydroxytryptamine, serotonin
AAV	Adeno-Associated virus
ACh	Acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
CBT	Core body temperature
CNS	Central nervous system
CPP	Conditioned place preference
CSDS	Chronic social defeat stress
DA	Dopamine
DSM-V	Diagnostic and statistical manual of mental disorders, 5th edition
DRN	Dorsal raphe nucleus
EEG	Electroencefalography
E/I	Excitatory/inhibitory
eEF2	Eukaryotic elongation factor 2
FDA	Food and drug administration
FrA	Frontal association area
FST	Forced-swimming test
GABA	*Gamma*-Aminobutyric acid
HAM-D	Hamilton Depression Rating Scale

HFO	High Frecuency Oscillations
HP	Hippocampus
IL	Infralimbic
IMAO	Monoamine oxidase inhibitors
i.p.	Intraperitoneally
LC	*Locus coeruleus*
MADRS	Montgomery-Åsberg Depression Rating Scale
MDD	Major depression disorder
MT	Melatonin
MT_1-/-	Melatonin 1 receptor knock-out
MT_2-/-	Melatonin 2 receptor knock-out
mTOR	Mammalian target of rapamycin
NA	Noradrenaline
NAc	Nucleus Acummbens
NMDA	N-methyl-D-aspartate receptor
PFC	Prefrontal cortex
PL	Prelimbic
PSD	Power spectral densities
SCN	Suprachiasmatic nucleus
SNRI	Selective noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor

SV	Synaptic vesicle
sVGLUT1	Vesicular glutamate transporter 1 silent mutant
Syn	Synapsin
TCA	Tricyclic antidepressant
TRD	Treatment Resistant Depression
TST	Tail suspension test
VGLUT	Vesicular glutamate transporter
VGLUT1+/-	Vesicular glutamate transporter 1 knock-down
WB	Western blot
WT	Wild type
YFP	Yellow fluorescent protein

Introduction

1. Facing major depression disorder

Major depression disorder (MDD) is a mood disorder primarily characterized by episodes of severe low mood and loss of interest or inability to experience pleasure in pleasant activities, a phenomenon that is called anhedonia (Bromet *et al.,* 2011). In addition to these core symptoms, different kinds of signs are combined in different intensity and time: emotional (sadness, blame, aggressiveness or suicidal tendencies) or biological (agitation, insomnia, loss of appetite) (Wong & Licinio, 2001). According to the Diagnostic and Statistical Manual of Mental Disorders (DMS-V 2013) of the American Psychiatric Association, the diagnoses of MDD is established when the patient presents three or four of those symptoms together with one of the core symptoms and persist for at least more than two weeks. MDD can be classified according to different criteria. According to the evolution of the disease it can be unipolar if there are only depressive episodes or bipolar when they alternate with obsessive states. A recurrent depressive disorder is the one in which depressive episodes are alternated. When symptoms have a mild but prolonged intensity lasting longer than two years, it is classified as dysthymic disorder. Treatment-resistant depression (TRD) is defined as a depression that does not respond to two or more different antidepressants drugs at adequate dosage and duration.

The World Health Organization (WHO) estimates that around 300 million people suffers from MDD and that it will be the second most prevalent cause of illness-induced disability by the year 2020. Since it represents 33% of the total cost for mental health, it is considered the most expensive mental disorder in Europe (Murray *et al.,* 1996; Ferrari *et al.,* 2013). Remarkably, five percent of the population suffers from this disease and one fifth of people experience one depressive episode through their life (Kessler *&* Murray*,* 2013). Moreover, MDD is one of the main triggers for suicide, being one of the leading causes of death worldwide accounting for nearly one million deaths each year. Unfortunately, suicide attempts are even more frequent (World Health Organization WHO, 2015; Ribeiro *et al.,* 2018). Taking these data into account, educational and health organizations have to develop social strategies to curb this disease.

2. Searching for pharmacological treatments

The research that eventually led to the development of the antidepressants began in the late 50s, when it was noted that drugs that inhibited the reuptake of monoamine neurotransmitters had antidepressant activity, through a mechanism that nowadays is

not totally discovered. The hypothesis was that these drugs increase mainly serotonin (5-HT) and noradrenaline (NA) in the synaptic cleft, being these increased levels the key of their effectiveness (Schildkraut, 1965).

2.1. Current antidepressants

Based on the monoamine hypothesis, pharmacologists were able to develop monoamine oxidase inhibitors (IMAO) and tricyclic antidepressants (TCA), which were the first monoaminergic antidepressants in use but which often had a side effects burden due to their relatively non-selective binding profile. After that, the first selective serotonin-reuptake inhibitors (SSRIs) were released in the late 80s including fluoxetine, paroxetine, citalopram and sertraline. These drugs along with selective norepinephrine-reuptake inhibitor (SNRIs) reboxetine or double 5-HT and NA reuptake inhibitors (venlafaxine and duloxetine) have remained as the first-line agents in the treatment of depression up to date (Thomas *et al.,* 2017).

Other clinical efficient drugs combine the action of monoamine reuptake inhibitors and modulators of other receptors such as mirtazapine (5-HT_{2A} and α_2-adrenergic receptor antagonists) or trazodone (5-HT_{2A} antagonist). Finally, vortioxetine as SSRI, 5-HT_3 antagonist and 5-HT_{1A} partial agonist has emerged as a novel therapy trying to improve SSRI action (Alvarez *et al.,* 2012).

In the last decade, an innovative antidepressant treatment has been proposed based on an attractive hypothesis linking depressive symptoms to circadian rhythm disruption and suggesting a key role for melatonin (MT) receptors (Bouwmans *et al.,* 2015). Agomelatine is the first melatoninergic agonist that activates MT receptors, MT_1 and MT_2, and restores circadian rhythms. Additionally, it antagonizes 5-HT_{2C} receptors, promoting monoaminergic transmission, especially in the prefrontal cortex (PFC). The efficacy, tolerability and safety of agomelatine has already been confirmed in several double-blind studies (Delagrange & Boutin 2006; Kennedy *et al*., 2008; Jockers *et al*., 2008; Guardiola-Lemaitre *et al*., 2014). Finally, a compound recently approved by the food and drug administration (FDA) for post-partum depression is brexanolone (alopregnenolone), a neurosteroid considered to be GABA-A receptor modulator.

Unfortunately, currently available interventions including monoamine-based pharmaceutical and psycho-behavioural therapies require several weeks to achieve beneficial effects (Blier, 2003; Machado-Vieira *et al*. 2010) and approximately more than one third of the patients undergoing this standard treatment do not respond being TRD patients (Insel *et al.,*2009; Rush *et al.,* 2006). In MDD the lack of immediate

clinical response, the prolonged period to achieve remission and the lack of response leading individuals to TRD, highlight the notion that currently available antidepressant treatments are inadequate for many MDD patients (Kadriu *et al.,* 2019). In addition, these treatments are often accompanied by undesirable side effects that often lead to drug withdrawal (Gelenberg *et al.,* 2000; Insel *et al.,* 2009). Therefore, there is an urgent need for better antidepressant medications, with a faster onset of action (Zanos *et al.,* 2018). In this sense the discovery of the rapid-acting antidepressant activity of N-methyl-D-aspartate receptor NMDA antagonists has moved the research beyond the monoamine hypothesis and has brought some hope for patients with TRD (Thomas *et al*., 2017).

2.2. Rapid acting antidepressants

Early in the 90´s, the potential rapid antidepressant action drugs were first shown (Trullas & Skolnick, 1990), by demonstrating the anti-helpless behaviour of MK-801 and AP-7 in the forced-swimming test (FST) (Skolnick *et al*., 2009). Later on, other molecules having a selective GluN2B NMDA antagonist action were developed such us Ro 25–6981 and it was proved to have rapid-antidepressant action in animal models (Skolnick *et al*., 2003; Li *et al.*, 2010; Jimenez-Sanchez *et al.,* 2014).

The NMDA antagonist ketamine

Ketamine, a chiral arylcyclohexylamine (R-S)- 2-(2-chlorophenyl)-2-methylaminocyclo-hexanone, was synthesized by Parke-Davis in 1963 and was first approved by the FDA in 70's as a dissociative anaesthetic agent. Ketamine is classified as an NMDA receptor channel blocker antagonist; although it's pharmacological profile is complex due to its affinity for numerous receptors. For instance, ketamine and its metabolites inhibit monoamines transport (Smith *et al.,* 1981) and increases 5-HT (Kinoshita *et al.,* 2018), NA (Kubota *et al.,* 1999a) and dopamine (DA) (Kokkinou *et al.,* 2018) in the PFC. Also, it has been reported to reduce neuronal responses to acetylcholine (ACh) by inhibiting nicotinic ACh receptor activation, while acting as an acetylcholinesterase inhibitor to raise brain ACh levels (Cohen *et al.,* 1974).

Later on, Krystal and colleagues (1994), found that a single 40 min intravenous infusion of a sub-anesthetic dose of ketamine (0.5 mg/kg), which produced light dissociative effects, resulted in significant improvement in depression symptoms within hours after administration of the drug. This robust antidepressant effect of ketamine occurred within 4 h post-administration, a time point when the dissociative effects were no longer

present, and lasted for 7 days on average in major depressed patients. Following this finding, other relevant double blinded clinical studies have replicated and further extended the findings of ketamine efficacy into patients with TRD using different doses and routes of administration such as oral and intranasal. Taking into account the most recent studies (Murrough *et al.,* 2013; Lapidus *et al.,* 2014; Singh *et al.,* 2016a; 2016b; Loo *et al.,* 2016; Su *et al.,* 2017) we could say that ketamine is an effective treatment for TRD patients, since more than half of the total patients achieved response, meaning they had a reduction equal or higher than 50% on their Montgomery-Asberg Depression Rating Scale (MADRS) or Hamilton Depression Rating Scale (HAM-D) score.

This finding has revolutionized and established the concept of rapid-acting antidepressant medications. Nevertheless, this drug is restricted to certain subgroups with suicidal ideation or TRD, and it requires close monitoring when it is administered, due to its side effects, that includes dissociation, psychotomimetic properties and abuse potential (Sanacora *et al*., 2017). Therefore, alternative medications that share ketamine robust antidepressant actions, but lack its side effect, are an urgently need.

Ketamine rapid-antidepressant actions are also supported at the preclinical level. Firstly, the anti-helpless behaviour of ketamine using the tail suspension test (TST), a test widely used for antidepressant drug *screening*, is well established (Steru *et al.,* 1985; Koike *et al*., 2011; Tang *et al.,* 2015). Moreover, it reverses anhedonia and other depressive related behaviours in depression models based on chronic stress, the chronic mild stress (CMS) and chronic social defeat stress (CSDS) models (Li *et al.,* 2010; Autry *et al.,* 2011 suplemental; Yang *et al.,* 2015)

The first steps for esketamine in the clinical ward

In March 5th 2019, the FDA approved intranasal esketamine (Spravato™) for adult patients with TRD (U.S. Food and Drug Administration. 2019) corresponding to the S enantiomer of ketamine (S-(+)-ketamine). S-(+)-ketamine has three to four times higher affinity to the NMDA receptor than R-(-)-ketamine (Vollenweider *et al.,* 1997). The enhanced pharmacological potency of esketamine suggests that the use of this enantiomer may reduce undesirable side effects without altering its clinical benefit treating depression (Paul *et al.,* 2009).

Esketamine use is restricted to TRD patients and treatment is received under supervision in a health center, not being possible to take the spray home. Patients are

under observation by a physician for at least 2 hours after they use the spray. The FDA recommends that patients should continue taking classic antidepressants. As with ketamine, caution is warranted with esketamine due to its dissociative symptoms and potential drug abuse liability (Freedman *et al.,* 2018). Yet, if this drug proves to be effective in post-market clinical follow-up studies, it might be worth to consider it a first-choice drug to be administered within the first weeks of a first depressive episode. Perhaps, in the near future, to find the right combined posology of esketamine or another similar non dissociative drug together with monoaminergic antidepressants might be the challenge of this 21st century, to make an important advance in the treatment of this illness.

Other NMDA antagonists

Following these promising findings with ketamine and esketamine other NMDA receptor antagonists have been developed. These promising new compounds have shown efficacy in several animal tests predictive of rapid-acting antidepressant actions and are now being tested in clinical phase for the treatment of TRD. Compounds currently in phase II include AV-101, an oral NMDA glycine B site antagonist and REL-1017 also called dextromethadone. In Phase III, AXS-05 is being studied, which combines a dextromethorphan, a NMDA receptor antagonist, with bupropion as a selective dopamine reuptake inhibitor.

3. Hypothesis on the neurobiology of depression

The biological factors that contribute to the development of depression remain unknown. This fact may be due in part to the complexity of the diagnosis of depression (Fried, 2017) and the difficulty to study pathological changes in the human brain and the limitations of post-mortem brain studies (Frewen *et al.,* 2008).

According to the classical monoaminergic hypothesis of depression, pharmacological treatment of depression is based on restoring levels of these neuromodulatory neurotransmitters 5-HT, NA and/or DA that are usually deficient in the MDD.

3.1. Monoaminergic hypothesis

The monoaminergic hypothesis was first proposed by Schildraut in 1965. This hypothesis postulates that MDD is driven by a lack of monoamines (mainly 5-HT and NA) in several regions of the brain. The fact that antidepressants produce an increase in these monoamines supports this theory. However, even though treatment with these medicines restores the levels of neurotransmitters immediately, the therapeutic effect

appears several weeks after drug administration. This fact leads to the hypothesis that what is necessary is an adaptation of the receptors located in the serotonergic and noradrenaline neurons (Artigas, 2013). Although the monoaminergic hypothesis is generally well accepted, a deficiency of monoamines seems to be insufficient to explain the course of MDD. Some reasons such as the ineffectiveness in TRD patients and the acquired vulnerability to relapse suggest that other important mechanisms are involved.

The serotonergic system

The serotonergic system is one of the most extensive and complex neurochemical networks in the central nervous system (CNS). It is estimated that the human brain contains about 250,000 5-HT neurons of a total of 10^{11} neurons (Jacobs & Azmitia, 1992; Artigas, 2013). Their axons originate mainly in the midbrain from the dorsal and median raphe nuclei. From the raphe nuclei, serotonin projections release 5-HT in different brain regions including PFC, hippocampus, pineal gland or hypothalamus. Importantly, different pathways such as glutamatergic neurons from layer V of PFC (Fink *et al.,* 1995), noradrenergic input from locus coeruleus (LC) (O'Leary *et al.,* 2007), GABAergic local interneurons in the dorsal raphe nucleus (DRN) (Varga *et al.,* 2001), and self-inhibition through 5-HT_{1A} autoreceptors exert a tight control over serotonin cell bodies in the raphe nuclei **(Figure 1)**.

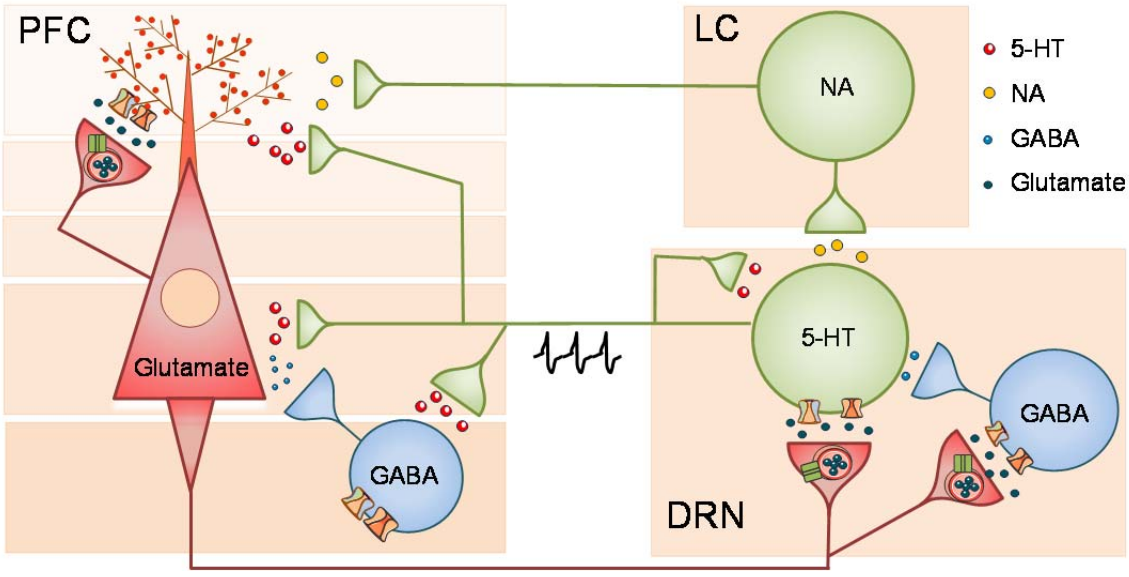

Figure 1. Representation of the functional connectivity between the prefrontal cortex (PFC) and brain stem containing, Locus coeruleus (LC) and the dorsal raphe nucleus (DRN).

Among the different serotonin receptors, the 5-HT_{1A} receptors exerts a key role on regulation of 5-HT function and therefore is involved both in depression and antidepressant action. This receptor is expressed in different regions of the mammalian brain: on 5-HT midbrain raphe nuclei neurons as an autoreceptor and as a postsynaptic receptor as well as in other forebrain areas such as the PFC, hippocampus, lateral septum, and hypothalamus (Azmitia *et al.,* 1996). The activation of the 5-HT_{1A} autoreceptor increases potassium conductance, thus hyperpolarizing the neuronal membrane exerting an inhibitory effect that leads to a reduced firing rate of the serotonergic neurons and 5-HT release (Chilmonczyk *et al.,* 2015). Postsynaptic 5-HT_{1A} receptors are also important. For instance, in PFC, it drives an inhibitory effect on glutamatergic pyramidal neurons, which, as previously stated, exert a long-loop feed-back mechanism of control over the 5-HT neuron activity both by direct innervation and via GABAergic interneurons (Sharp *et al.,* 2007).

Moreover, it is well known that the 5-HT_{1A} autoreceptor is deeply involved in the mechanism of antidepressant drugs. TCAs, IMAOs and SSRIs produce a remarkable increase of extracellular 5-HT in the raphe nuclei (Celada & Artigas, 1993). As a consequence, 5-HT_{1A} autoreceptors reduce cell firing and 5-HT release (Blier & de Montigny, 1994). However, the effectiveness of this negative feedback pathway declines after chronic treatment because elevated 5-HT in the raphe is able to desensitize the autoreceptor (Gardier *et al.,* 1996). Consequently, chronic antidepressants induce an increase in extracelular 5-HT levels in the forebrain.

The noradrenergic system

The noradrenergic system has also an important role in MDD (Chandley *et al.,* 2012). Principal neurons located in *locus coeruleus* (LC) in the midbrain are an important component of the body's neurobiological response to stress, but also related to other functions like arousal of the sleep-wake cycle. This nucleus is composed by neuromelanin, a dark pigment formed by the polymerization of NA. It is estimated that the human brain contains about 22,000 to 51,000 total pigmented neurons in the LC, very small amount if it is compared with the whole brain neuronal content (Mouton *et al.*, 1993). Nevertheless, NA projections to forebrain and limbic system (amygdala and hippocampus), are very important in the cognitive control processes such as attention/concentration, decision-making, memory, which, are deregulated in MDD. Moreover, descending glutamatergic pathways originated in the PFC control noradrenergic activity in LC (Jodo, & Aston-Jones, 1997; Chandley *et al.,* 2014; Chandler *et al.,* 2014). In addition, the influence of the noradrenergic system over the

serotonergic system is also very relevant. For instance, α_1 adrenoceptors in the DRN are stimulated by NA during resting conditions suggesting that LC has a positivive control of serotonin firing (Bortolozzi & Artigas, 2003) **(Figure 2)**.

The dopaminergic system

The dopaminergic system is also important to understand MDD. Anhedonia is considered a core feature of major depressive disorder, and the dopamine system plays also a pivotal role in this symptom (Belujon & Grace, 2017). Dopaminergic neurons (DA-producing nerve cells) are a total of 400,000 in the human brain (Schultz, 2007) and are located in different brain areas that control important roles like executive functions, motor control, motivation and reward. As part of the reward system, DA is synthesized in the ventral tegmental area (VTA) and releases DA to different areas such as PFC, hippocampus, amygdala and the nucleus accumbens (NAc) **(Figure 2)**. Research using neuroimaging, pharmacological, and electrophysiological methods in humans and animal models of depression have demonstrated dopaminergic dysfunctions in this disease (Friedman, et al., 2008).

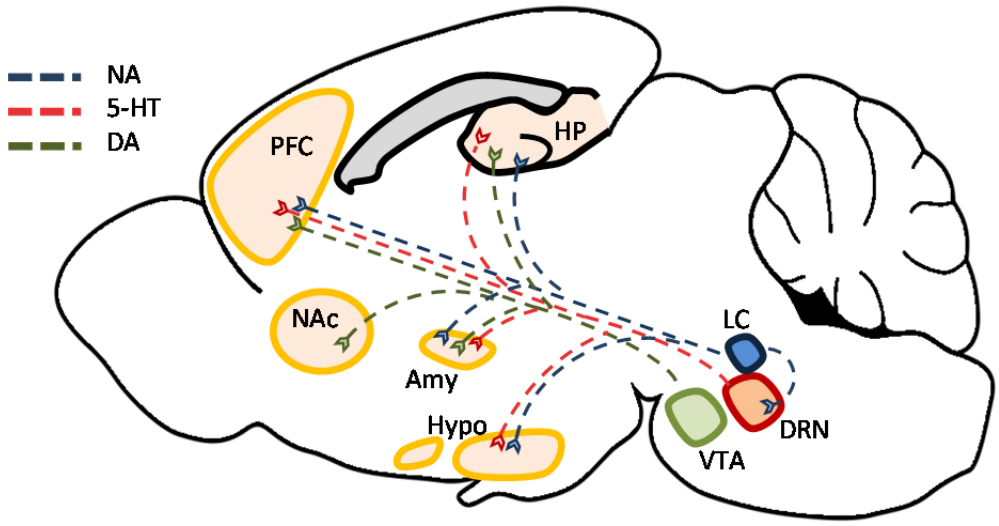

Figure 2. Principal innervated areas by serotonin, noradrenaline and dopamine from the dorsal raphe nuclei (DRN), locus coeruleus (LC) and ventral tegmental area (VTA) respectively. Prefrontal cortex (PFC), Nucleus Acummbens (NAc) Amygdala (Amy), Hypothalamus (Hypo) and Hippocampus (HP).

Collectively, these studies demonstrate that 5-HT and NA mainly, together with dopamine, play a prominent role in antidepressant drug action. Although, the monoaminergic hypothesis itself does not provide a complete explanation for MDD it is still the most accepted hypothesis. However, scientists keep struggling to elucidate other mechanisms that can provide a deeper understanding of the disease.

3.2. The circadian rhythm of melatonin

Over the past 50 years it has become increasingly clear that melatonin deficiency plays a key role in the pathophysiology of MDD. In particular disturbed melatonin secretion rhythms from the pineal gland, including advanced phases and/or decreases in nocturnal amplitude of melatonin rhythm have been observed (Hickie *et al.,* 2011; Bouwmans *et al.,* 2015). In addition, this "low melatonin syndrome" has been associated to the circadian disruption of the sleep/wake cycle, body temperature (Avery *et al.,* 1997; Cajochen *et al.,* 2003), cortisol secretion (Hickie *et al.,* 2011) and variations in mood symptom severity (Monteleone *et al.,* 2008). On the other hand, antidepressant effectiveness of melatonin has been associated to its chronobiotic properties (Lanfumey *et al.,* 2013). Indeed, melatonin results in an effective option for individuals with depressive symptomatology comorbid with circadian rhythm sleep disorder (Rahman *et al.,* 2010; Hickie *et al.,* 2011).

Melatonin is a neurohormone always secreted at night in both day and night-active species. It plays a regulatory role in a wide range of important physiological and behavioral processes, such as the sleep-wake cycle, thermoregulation, hormonal secretion, seasonal adaptation, reproduction and many others (Schulz *et al.,* 2009). It has also antidepressant, neuroprotective, anti-inflammatory and antioxidant effects (Boyce *et al.,* 2003; Wongprayoon *et al.,* 2017).

Through binding to melatonin membrane G-protein-coupled receptors 1 and 2 (MT_1 and MT_2) (Lacoste *et al.,* 2015) in the suprachiasmatic nucleus (SCN) of the hypothalamus, melatonin controls the expression of multiple clock genes (Nagy *et al.,* 2015), which exert a precise and stable molecular regulation of circadian rhythms. In human beings, melatonin, core body temperature (CBT) and cortisol rhythms are highly correlated within an individual subject (Shanahan *et al.,* 1997; Rajaratnam *et al.,* 2001; Czeisler *et al.,* 2010). For instance, the rising phase of the melatonin secretion rhythm highly correlates with the declining phase of the body temperature rhythm being, between 4 and 6 a.m., melatonin at maximum and CBT at a minimum (Czeisler *et al.,* 2010). In night-active species, like rodents, melatonin secretion and temperature rhythm go in parallel (Nakahara *et al.,* 2003; Weinert *et al*., 2007; Knight *et al.,* 2013).

These studies suggest the relevance to study in depth the role of each melatoninergic receptor subtype in depressive-like behaviors and circadian rhythm in order to provide a better comprehension on the possible involvement of these receptors in depression associated to circadian rhythm disruption.

3.3. The neurotrophic hypothesis

It has been shown by the literature that neurotrophic factors, most notably the brain derived neurotrophic factor (BDNF), has a role in MDD. Decreased expression and function of BDNF in PFC and hippocampus, is fundamental for a proper maintenance of synaptic connections. Patients with MDD have shown decreased levels of BDNF in brain (Altar *et al.,* 1997) and serum (Palomino *et al.,* 2006; Sen *et al.,* 2008). Also, an animal genetic model of deficient BDNF has been used as a valid model of depression (Lindholm & Castrén, 2014). In addition, it has been observed that direct infusion of BNDF into the brain produces an antidepressant effect and potentiates the efficacy of antidepressant treatment (Castrén & Rantamäki, 2010). Moreover, BDNF receptor called Tropomyosin receptor kinase B (TrkB), has been positively correlated with antidepressant efficacy, possibly through their roles in synaptogenesis and neurogenesis (Duman & Monteggia, 2006; Leal *et al.,* 2014). In line with this hypothesis, several studies have shown that BDNF mediate the therapeutic action of antidepressants (Berton *et al.,* 2006; Castrén *et al.,* 2007). Interestingly, chronic, but not acute, antidepressant treatment induces BDNF expression and immunoreactive fibers in the hippocampus and PFC of rodents (Nibuya *et al.,* 1996; De Foubert *et al.,* 2004). Conversely, it has been suggested that the delay of the antidepressant effect is due to the time necessary to produce neuroadaptive mechanisms that can improve neuronal plasticity (Kozisek *et al.,* 2008; Pittenger & Duman, 2008; Björkholm & Monteggia, 2016). In keeping with this, injection of ketamine has shown to increase the levels of BDNF and pro-BDNF 30 minutes after drug injection (Garcia *et al.,* 2008; Autry *et al.,* 2011; Lepack *et al.,* 2016). BDNF has also been shown to activate the mTOR signaling pathway (Nosyreva *et al.,* 2013), which is involved in protein synthesis and increased excitatory neurotransmission (Miller *et al.,* 2014).

3.4. The glutamatergic hypothesis

Several studies have shown a glutamatergic and GABAergic deregulation in depression in the PFC (Robbins & Murphy *et al.,* 2006). The PFC is mainly composed by pyramidal cells and is one of the main areas involved in MDD (Murrough *et al.,* 2016). This cells control big amount of different processes such as cognitive behavior, personality, decision making, executive function, attention, learning and memory. It is estimated that about 80% of neurons in neocortex are spiny and excitatory, while about 20% of the neurons are smooth and inhibitory (Douglas and Martin, 2007). Glutamate is the starting substrate for the biosynthesis of GABA, a process catalyzed by the enzyme glutamate acid decarboxylase which is altered in several psychiatric diseases

(Akbarian *et al.,* 1995). Functional magnetic resonance imaging has also demonstrated decreased cortical levels of GABA (Sanacora *et al.,* 2004). Among different glutamate targets altered in MDD, special attention should be paid to changes in expression/function of specific NMDA receptor subunits (Feyissa *et al.,* 2009).

Role of NMDA and AMPA receptors in antidepressant action

NMDA receptors are glutamatergic ion channel receptors, and are composed of three different subunits that may be derived from seven different subunit genes: GluN1, GluN2A-D, and GluN3A-B (Vyklicky *et al.,* 2014). NMDARs exhibit remarkable properties that distinguish them from other types of ligand-gated ionotropic receptors. First, their ion channel is subject to a voltage-dependent block by Mg^{2+}; second, NMDAR channels are highly Ca^{2+} permeable; third, they display unusually slow kinetics owing to slow glutamate unbinding; fourth, their activation requires the presence not only of glutamate but also of a co-agonist (glycine or d-serine); and fifth, they are equipped with an array of modulatory sites conferring an exquisite sensitivity to the extracellular microenvironment **(Figure 3)**.

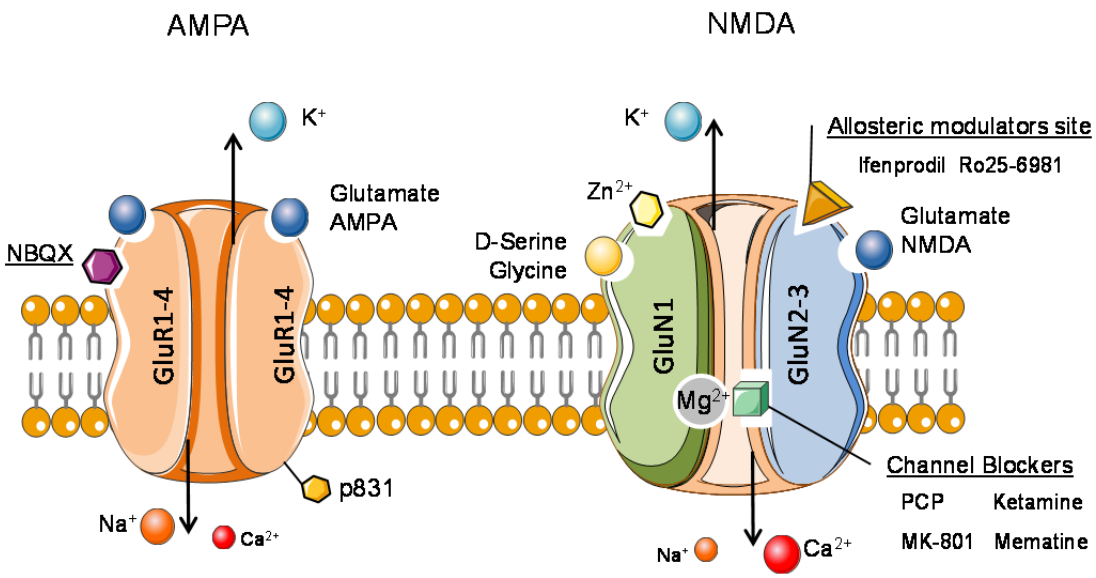

Figure 3. AMPA and NMDA receptors structure, subunits and compounds exerting their action on modulatory sites.

NMDA receptors are molecular mediators of plasticity and among the different subunits GluN2B is particularly important to plasticity (Shipton & Paulsen, 2013). Current evidences suggest that the presence of GluN2B subunit-containing NMDARs at the postsynaptic density might be necessary for the synaptic plasticity strengthening. Specifically, GluN2B interact with calcium/calmodulin-dependent protein kinase II

(CaMKII) and mediate AMPA receptors insertion through phosphorylation of Ser831 on GluA1 subunit (Feyissa *et al.,* 2009; Halt *et al.,* 2012; Shipton and Paulsen, 2013). Interestingly, the GluN2B-containing NMDARs are proposed to underlie the antidepressant effects of ketamine (Burgdorf *et al.,* 2013).

AMPA receptors are ionotropic transmembrane receptors for glutamate that mediates fast synaptic transmission in the CNS and are composed by four types of subunits, designated GluA1-4. Changes in AMPA glutamate receptors (AMPARs) play a major role in the expression of plasticity at excitatory synapses. Enhancement of synaptic glutamate transmission is largely mediated by an increase in the number and/or conductance of post-synaptic AMPA receptors in the membrane (Shepherd & Huganir, 2007; Henly & Wilkinson, 2016). The role of AMPA receptors-mediated plasticity in rapid antidepressant actions is a topic of great interest nowadays.

In particular, activation of AMPA receptors in the PFC has been linked to antidepressant like effects (Li *et al.,* 2010; Koike *et al.,* 2011). Importantly, rapid antidepressant action of NMDA receptor antagonists has been linked to transient activation of AMPA receptors (Maeng *et al.,* 2008; Koike *et al.,* 2011; Zanos *et al.,* 2016). In line with this, chronic treatment with SSRIs and other antidepressants enhance glutamate transmission and synaptic strength in the PFC by activation of AMPA receptors (Koike *et al.,* 2011; Wolak *et al.,* 2013). Specifically, an increase in the phosphorylated form of the GluA1 subunit (*p*-GluA1) at Ser831 is responsible for the stimulation of AMPA receptors.

Noteworthy, a reciprocal interaction between glutamate and serotonin was early described in the PFC. Activation of glutamate receptors in the PFC has been linked to antidepressant-like effects (Li *et al.,* 2010, Koike *et al.,* 2011). Further, in this region, NMDA receptors have been shown to stimulate serotonin release from fibers originating in the raphe nuclei (Fink *et al.,* 1996). Similarly, serotonin in the PFC enhances glutamate transmission acting through postsynaptic 5-HT_{2A} receptor activation (Aghajanian & Marek, 1999). Specifically, it has been shown that optogenetic or local glutamate stimulations of the PFC evoke rapid antidepressant-like effects (Fuchikami *et al.,* 2015; Jimenez-Sanchez *et al.,* 2016). Likewise, monoaminergic antidepressants enhance glutamate excitatory transmission specifically in the PFC (Koike *et al., 2*011; Mørk *et al.,* 2012; Wolak *et al.,* 2013; Riga *et al.,* 2016).

Vesicular glutamate transporters, key regulators of glutamate release

Synaptic vesicle (SV) loading with neurotransmitter is an important parameter for the fine-tuning of neurotransmission. Glutamate secretion at excitatory synapses is tightly regulated to allow a precise tuning of synaptic strength. Vesicular Glutamate Transporters (VGLUTs) accumulate glutamate into SV and thereby regulate quantal size.

Three isoforms of VGLUTs (VGLUT1-3) have been identified. Different VGLUTs are very similar with respect to substrate specificity, kinetics, and pharmacology. These transporters mediate glutamate uptake into SV and are driven by a proton electrochemical gradient generated by the vacuolar H-ATPase (Bellocchio *et al.,* 2000). VGLUT1 is the major isoform of the cerebral and limbic cortex, where it is selectively located on SV of excitatory glutamatergic terminals (Fremeau *et al.,* 2001). VGLUT2 is the major isoform on the diencephalon, brain stem and spinal cord; low levels are expressed in PFC (Liguz-Lecznar & Skangiel-Kramska, 2007). The third isoform VGLUT3 defines a discrete subpopulation of glutamatergic neurons and is also co-expressed in cholinergic, serotonergic (Fernandez *et al.,* 2015) and even GABAergic neurons (Herzog *et al.,* 2004).

Genetic inactivation of VGLUT1 drastically reduces glutamatergic neurotransmission in cortical and hippocampal neurons with a specific reduction in quantal size seen in cultured hippocampal neurons from VGLUT1 knock-out mice, leading to severe malfunctions and lethality (Wojcik *et al.,* 2004). The modulation of its activity or expression is implicated in the pathophysiology of several neurological and psychiatric diseases including schizophrenia (Oni-Orisan *et al.,* 2008), Alzheimer's disease (Kashani *et al.,* 2007), Parkinson's disease (Kashani *et al.,* 2008) and epilepsy (Juge *et al.,* 2010). Moreover, cortical, frontal and hippocampal circuits, in which VGLUT1 positive excitatory neurons are central, play an operational executive key role in integrating affective imprints and cognitive processes (Tordera *et al.,* 2007). Glutamatergic neurons from PFC are suggested to modulate 5-HT activity in the DRN via VGLUT1, acting indirectly through GABAergic interneurons (Hajos *et al.,* 1998; Varga *et al.,* 2001) and controlling 5-HT release (Chandley *et al.,* 2014). Clinical studies have shown that decreased levels of VGLUT1 could be a potential biological risk factor of MDD (Uezato *et al.,* 2009; Gilabert-Juan *et al.,* 2012). Also, different preclinical studies have shown the relevance of VGLUT1 in depressive-like behaviors such anhedonia, stress or learned helplessness (Tordera *et al.,* 2007; Elizalde *et al.,* 2010; Brent Myers *et al.,* 2017).

Moreover, VGLUT1 levels are modulated after a chronic treatment with different classic antidepressant treatment, such as fluoxetine, paroxetine or imipramine (Tordera *et al.,* 2005) and with different rapid antidepressants such as ketamine (Ren *et al.,* 2015; Lisek *et al.,* 2017), MK-801 (Farley *et al.,* 2012) or scopolamine (Yu *et al.,* 2017).

Role of the mammalian Target of Rapamycin (mTOR) pathway in antidepressant action

Stimulation of mammalian target of rapamycin complex 1 (mTOR) is a large serine/threonine kinase that regulates the initiation of protein translation and increases synaptic protein synthesis. A study from Li *et al.,* (2010) found for first time that the activation of mTOR in PFC underlies the mechanism of ketamine exerting a rapid antidepressant effect. Induction of mTOR signaling occurs within 30 minutes after ketamine administration and is transient, with the mTOR phospho-proteins returning to basal, non-stimulated levels by 3h (Adaikkan *et al.,* 2018). It is demonstrated that an inverted U dose response for ketamine, with low doses (5 and 10 mg/kg) stimulating and a higher, anesthetic dose (80 mg/kg), having no effect on mTOR signalling (Li *et al.,* 2010). After this finding, some studies have shown that chronic SSRIs also estimulate mTOR pathway (Park *et al.,* 2014; Liu *et al*., 2015; Xu *et al.,* 2018).

After ketamine, enhanced BDNF release and activation of TrkB trigger downstream pathways via an activation of the phosphatidylinositol 3-kinase (PI3K) or the mitogen-activated protein kinase MAPK/ERK signalling pathway. Both pathways promote protein synthesis through activation of the mTOR (Yoshii *et al.,* 2010). Moreover, pre-treatment with inhibitors of these two kinases, completely blocked the ability of ketamine to stimulate the phosphorylation of mTOR and P70S6 kinase (Li *et al.,* 2010). Further, downstream mTOR pathway, phosphorylation of the synaptic P70S6 kinase (P70S6k), and S6 ribosomal subunit regulates neurogenesis and dendrite spine growth (Dwyer *et al.,* 2015).

Role of the eukaryotic elongation factor 2 in antidepressant action

The eukaryotic elongation factor 2 (eEF2) has two states (phosphorylated and dephosphorylated) in the cell. eEF2 kinase (eEF2k) causes an inactivation (phosphorylation) of its substrate protein, eEF2 (Thr 56), leading to the blockade of the elongation phase of protein synthesis and thus inhibition of protein translation (Park *et al.,* 2008). Activation of NMDA receptors activates eEF2k, leading to increased

phosphorylation (and inhibition) of elongation factor 2 (eEF2) and overall decreased protein translation (Scheetz *et al.,* 2000). Interestingly, ketamine is proposed to block synaptic NMDAR-mediated spontaneous activation of eEF2k, thereby causing a de-phosphorylation of eEF2 and a consequent activation of protein synthesis and enhancement of BDNF translation (Autry *et al.,* 2011). This hypothesis is supported by the finding that eEF2k knockout mice present resistant responses to ketamine using the forced-swim test (FST) (Nosyreva *et al.,* 2013; Adaikkan *et al.,* 2018).

Altogether, three possible molecular mechanisms for the rapid antidepressant action of ketamine have been proposed (Zanos and Gould, 2018) **(Figure 4)**. The first molecular mechanism **(1)** is based on elevation of glutamate levels by ketamine in the PFC which could occur indirectly through NMDA receptors blockade in GABA interneurons (Su *et al.,* 2018). Another possible mechanism **(2)** could be the blockade of NMDA receptors which increases fast glutamate signalling trought AMPA receptors leading to an increase in intracellular signals and protein synthesis (Li *et al.,* 2010). Finally, **(3)** ketamine-induced blockade of NMDA receptors inhibits eEF2, leading to eEF2 de-phosphorylation and subsequent activation of BDNF translation (Autry *et al.,* 2011).

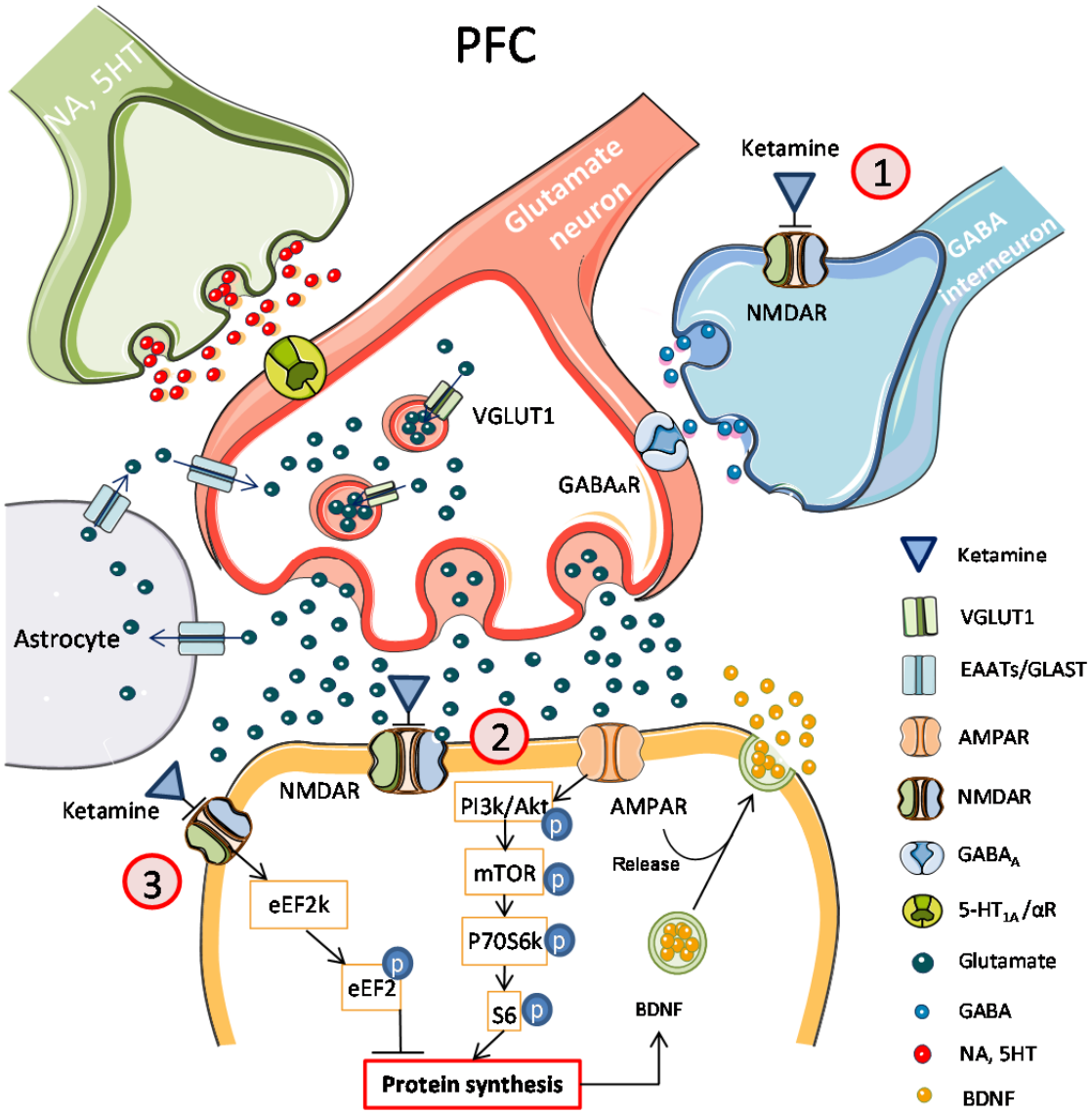

Figure 4. Schematic representation of proposed mechanism for the antidepressant action of ketamine a synapse in PFC after an injection of sub-anesthetic dose of ketamine. Adapted figure from Zanos and Gould, (2018).

4. Electrophysiological cortical oscillatory activity in major depression and animal models

In vivo electrophysiology has been crucial for elucidating important properties of many neural systems. EEG has the ability to reveal voltage events and oscillations emanating from and within the brain in characteristic frequency bands, which have been given names such as delta (δ; 0-4 Hz), theta (θ; 4–8 Hz), gamma (γ; 30–100 Hz) and high-frequency oscillations (HFO; 100–200 Hz). Relatively little is known about their *in vivo* electrophysiological effects on the human brain.

In patients, some studies have found that subjects with high depression scores had reduced resting gamma in the anterior cingulate cortex (Pizzagali *et al.,* 2006; Liu *et al.,* 2014). Moreover, low-frequency EEG activities including delta and theta power has been shown to be altered in MDD patients (Knott *et al.,* 2000). In addition, these alterations can be reverted by antidepressant treatment in recovered patients (Iosifescu *et al.,* 2009; Baskaran *et al.,* 2012; Fitzgerald & Watson, 2019).

Oscillatory signals are indeed an effective tool for elucidating circuit-specific neural mechanisms through which antidepressants produce their therapeutic effects in animal models of depression (Dzirasa *et al.,* 2013; Nagy *et al.,* 2016). Interestingly, noradrenaline-selective drugs like reboxetine and desipramine enhance theta and gamma oscillations (Hajós *et al.,* 2003). Moreover, acute systemic low-dose administration of ketamine tends to enhance gamma and high frequency oscillations (HFO) in a number of cortical and subcortical structures, and could be related to fast alteration of glutamatergic systems in mood-related circuits (Lazarewicz *et al.,* 2010; Nicolás *et al.,* 2011; Olszewski *et al.,* 2013; Shaw *et al.,* 2015; Skoblenick *et al.,* 2016).

Hypothesis and Objectives

Major depression is a mental disorder characterized by extreme low mood and anhedonia that show very often together with many other psychological and/or physiological disturbances. This illness can cause moderate to severe alterations in the patient daily life, in many aspects of work and personal areas. In recent years, major depression is turning into one of the leading causes of disability worldwide. For instance, it is estimated that five percent of the population suffers from this disease and one fifth of people experience one depressive episode through their life. Therefore, facing major depression is an urgent social and medical need since it involves a lot of suffering in patients and their families or friends as well as an important socio-economic cost worldwide.

Depression has been studied for many years at preclinical and clinical level in order to identify molecular mechanisms underlying the disease and to find better pharmacological treatments. The monoaminergic hypothesis that links monoamine levels (mainly 5-HT and NA) to depression and antidepressant action has been the leading thesis for more than 50 years. However, this hypothesis has important limitations including the latency time needed for antidepressants to start working, the great number of resistant patients and the enduring vulnerability of many patients to relapse. Moreover although the safety of current antidepressants has improved they are not free of side effects.

Those limitations drive the need to develop new strategies to treat this mental disorder. A growing number of studies are now reporting alterations in glutamate transmission in the PFC and a therapeutic potential of glutamate targets in depressive disorders. The PFC, mainly involved in decision-making and executive functions, is also an essential component of the neural circuitry modulating stress responses and emotions. In recent years, one of the molecules that have opened this field is the NMDA antagonist ketamine that increases glutamate transmission in the PFC and is believed to exert a rapid synaptic reconfiguration of this impaired area in the patients. This molecule has the ability to enhance glutamate transmission and trigger antidepressant effects within a few hours being *de novo* synthesis of BDNF and the NMDA dependent transient activation of AMPA receptors involved in this antidepressant action.

The finding of NMDA antagonists as rapid-acting antidepressant medications has revolutionized the approach to this illness. Importantly, in March 5th 2019, the FDA approved intranasal esketamine (Spravato™) for adult patients with TRD (U.S. Food and Drug Administration. 2019). Yet not all the TRD patients respond to ketamine and in addition, this drug requires close monitoring when it is administered, due to its side

effects that include dissociation, psychotomimetic properties and abuse potential. However, if this drug proves to be effective in post-market clinical follow-up studies, it will be an important advance of this 21st century and will promote the searching of alternative medications that share ketamine robust antidepressant actions, but lack its side effect.

At the preclinical level, most studies focused on identification of molecular mechanisms mediating the rapid antidepressant response of ketamine have been carried out in healthy mice. In contrast, genetic models of depression with a specific molecular modification, present specific depressive-like endophenotypes enriching the scenario to test the pharmacological action of classic and new antidepressant drugs. Given that ketamine stimulates glutamate signalling, a genetic model of impaired glutamate function would provide a better comprehension of the molecular mechanisms mediating antidepressant response or resistance to treatment.

Previous studies carried out in our lab demostrate that VGLUT1+/- heterozygous mice show depressive-like behavior. Interestingly, variations in VGLUT1 levels affect to the glutamate vesicular content and to synaptic availability of glutamate. Specifically, we aim to answer to the question whether ketamine is able to raise glutamate function in this model and initiate a coordinated cascade that mediates a change in neural plasticity relevant to antidepressant action. Our first approach was to characterize the molecular and behavioural effects of classic monoaminergic antidepressant like the selective noradrenaline reuptake inhibitor reboxetine. Subsequently, we observed that VGLUT1+/- mice proved to be resistant to ketamine, and thus, we investigated strategies directed to rescue the lack of effect of ketamine in these mice. Firstly, we pretreated the mice with reboxetine to induce a *priming* effect in this model before ketamine injection. A second strategy consisted on the use of recombinant adeno-associated virus (AAV) technology to induce VGLUT1 expression specifically in the PFC of the heterozygous.

Besides, another genetic model in which glutamate function is not involved *a priori* was selected. Specifically the melatonin 2 receptor deficient model is associated to circadian rhythm disruption. This model is based on the increasing knowledge over the past 50 years that some depressed patients show disturbed melatonin secretion. Through binding to melatoninergic receptors, MT_1 and MT_2, in the suprachiasmatic nucleus (SCN), melatonin exerts a precise and stable molecular regulation of circadian rhythms. In this model, we first studied the role of each melatoninergic receptor subtype in the regulation anhedonia, one of the core symptoms of depression, together

with circadian temperature. Secondly, we tested the antidepressant action of ketamine in this model.

Specifically, using the VGLUT1+/- genetic model we aimed to study:

1. **The role of VGLUT1 in depressive-like phenotype and antidepressant action.** Particularly, we have studied:

1.1. The depressive-like behavior of a wide population of VGLUT1+/- mice. In particular anhedonia and helpless behavior were studied in the sucrose intake test and in the tail suspension test. Further the correlation between depressive-like behavior and VGLUT1 levels was studied.

1.2. The effect of chronic treatment with reboxetine in the depressive-like behaviour of VGLUT1+/- mice compared to their WT littermates.

1.3. The effect of reboxetine treatment in the activation of glutamate receptor dependent intracellular signalling linked to antidepressant action, in VGLUT1+/- mice compared to their WT littermates.

2. **Behavioural and molecular effects of ketamine in the VGLUT1+/- genetic model.** In particular, we have studied:

2.1. The effect of ketamine in the depressive-like behaviour of VGLUT1+/- mice compared to their WT littermates.

2.2. The effect of ketamine in the activation of glutamate receptor dependent intracellular signalling linked to antidepressant action in VGLUT1+/- mice compared to their WT littermates.

2.3. The effect of ketamine in the oscillatory activity in the cortex of VGLUT1+/- mice compared to their WT littermates.

3. **Rescue of the rapid antidepressant action of ketamine in the VGLUT1+/- mice.** Specifically, we have studied:

3.1 Behavioural and molecular effects of ketamine in the depressive-like phenotype of VGLUT1+/- model pretreated with reboxetine.

3.2. Behavioural effects of ketamine in the depressive-like phenotype of the VGLUT1+/- model with induced expression of VGLUT1 in the PFC using recombinant AAV technology.

Besides, in the MT_2-/- model we aim to study:

4. The role of MT2 deletion in depressive-like behavior associated to circadian temperature disruption. Specifically, we have studied:

4.1. The depressive-like behavior and circadian core body temperature (CBT) of MT_2-/- mice compared to MT_1-/- and WT littermates. In addition, the effect of reward exposure on nocturnal circadian CBT of these mice was studied.

4.2. The stress vulnerability MT_2-/- mice to develop depressive-like behaviors compared to MT_1-/- and WT littermates.

4.3. The effect of reboxetine treatment on depressive-like behavior and circadian CBT of MT_2-/- mice. In addition, the effect of reward exposure on nocturnal circadian CBT of these mice was studied.

4.4. Effect of the rapid-acting antidepressant ketamine in the depressive-like phenotype of MT_2-/- model.

Experimental Design and Methods

1. Animals

Heterozygous VGLUT1 (VGLUT1+/-) and wild-type (WT) male C57BL/6 mice (8 to 12 weeks old) bred in the animal house of the University of Navarra from heterozygous fathers (kindly donated by Dr. S. Wojcik, Gottingen, Germany) and WT mothers (Harlan, France) were used. Mice were weaned and genotyped at the age of 3 weeks. Heterozygous mice exhibited no apparent phenotypic abnormalities during development and adulthood.

MT_1 (MT_1-/-) and MT_2-deficient (MT_2-/-) C57BL/6 male mice, bred onto a melatonin-proficient 129/SvEv-C57BL/6 genetic background (Taconic, 8–10 weeks of age) were used (Benleulmi-Chaachoua *et al.,* 2018). No apparent phenotypic abnormalities and no significant differences among genotypes were observed in body weight gain over time in our facilities. Male CD1 retired breeders (Charles River, 5 months old) were used as the dominant strain for the CSDS procedure.

Every effort was made to minimize the number of animals used and their suffering. Food and water were available *ad libitum* for the duration of the experimental procedures unless otherwise specified. Animals were maintained in a temperature (21±1°C) and humidity-controlled room (55 ± 2%) on a 12-h light–dark cycle (lights on at 8:00 h). Experimental procedures were conducted according to the principles of laboratory animal care as detailed in the European Communities Council Directive (2010/63/EC), Spanish legislation (Royal Decree 53/2013) and approved by the Ethical Committee of University of Navarra.

2. Drug treatment

The classic antidepressant noradrenaline reuptake inhibitor reboxetine-HCl (kindly donated by Servier SL, Paris, France) was dissolved in saline (0.9%) and administered intraperitoneally (i.p.) at 15 mg/kg respectively once daily (at 10 a.m.) for three or four weeks. This dose has shown to effectively block noradrenaline reuptake (Hajos *et al.,* 2004) and antidepressant activity (in the FST) in acute studies (Palucha-Poniewiera *et al.,* 2017). The rapid acting antidepressant NMDA antagonist ketamine HCl (Anesketin® 100 mg/ml, Dechra, UK) was diluted in saline (0.9%) and administered (at 10 a.m.) at 5, 10 or 15 mg/kg, i.p. Repeated treatment included an injection every four days following a previous similar design (Zhang *et al.,* 2015). In rescue studies ketamine was administered i.p. at 10 mg/kg.

The 5-HT_{1A} receptor agonist 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) (RBI, Wayland, MA, U.S.A) was dissolved in saline and administered subcutaneously (s.c.) in a single dose of 0.25 mg/kg (García-García *et al.,* 2013).

Mice were anesthetized by i.p. injection of ketamine 75 mg/kg, (Ketamidor® Richterpharma, Wels, Austria) and xylacine 11mg/kg (Xilagesic®, Barcelona, Spain) for stereotaxic procedures. Buprenorphine (Buprex®, Schering-Plough, New Jersey, USA) was injected subcutaneously in a concentration of 0.1 mg/kg for reducing the nociception. Pentobarbital (Sigma- Aldrich, St. Louis, MO, USA) was dissolved in saline (0.9%) and administered i.p. at 40 mg/kg for general anesthesia for posterior perfusion for brain imaging experiments.

3. The VGLUT1+/- genetic model of depression: Experimental designs

Experimental design 1. Behavioral and molecular effects of the antidepressant reboxetine

We studied the effect of the classic antidepressant reboxetine in the depressive-like phenotype of VGLUT1+/-mice. Following two weeks intake baseline for the sucrose intake solution was established, WT and VGLUT1+/- mice were subdivided into vehicle and reboxetine groups being no difference in sucrose intake measures between subgroups prior to the treatments. Reboxetine (15 mg/kg, i.p.) or saline were administered daily along 3 weeks. The following tests were performed: locomotor activity (day 1) and marble burying (day 9) were studied 60 minutes after the drug injection. Also, hypothermia induced by 8-OH-DPAT was analyzed 30, 60 and 90 minutes after drug injection (day 18). Finally, on the last day of treatment (day 22), tail suspension test (TST) was conducted 60 minutes after the drug injection and then the fresh tissue was collected to study the protein expression levels in the brain PFC and in the DRN. Tests were performed from 9:00-1:00 p.m. **(Figure 5)**.

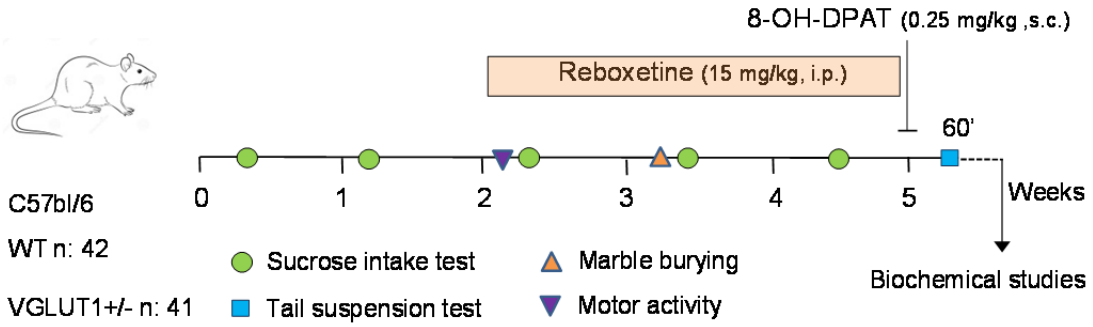

Figure 5. Experimental design followed to test the effect of the classic antidepressant reboxetine in the VGLUT1+/- depression model.

Experimental design 2. Behavioral and molecular effects of ketamine

In the next experiment, we studied the effect of the rapid acting antidepressant ketamine, already studied in the literature in other depression models (Yang *et al.,* 2016; Papp *et al.,* 2017). Three independent experiments using a different dose of ketamine were carried out:

Following three weeks in which the baseline intake for the sucrose intake solution was established, mice were treated with three different doses of ketamine (5, 10 or 15 mg/kg, i.p.) separated each one for four days. During treatment, sucrose intake test was performed for two weeks in order to analyze the anhedonic behavior. In adittion, locomotor activity (day 1), marble burying (day 9) were analyzed 60 minutes after drug injection. Finally, on the last day of treatment (day 13), TST was carried out 60 minutes after drug injection and then fresh tissue from the PFC was collected to analyze different protein expression levels **(Figure 6).**

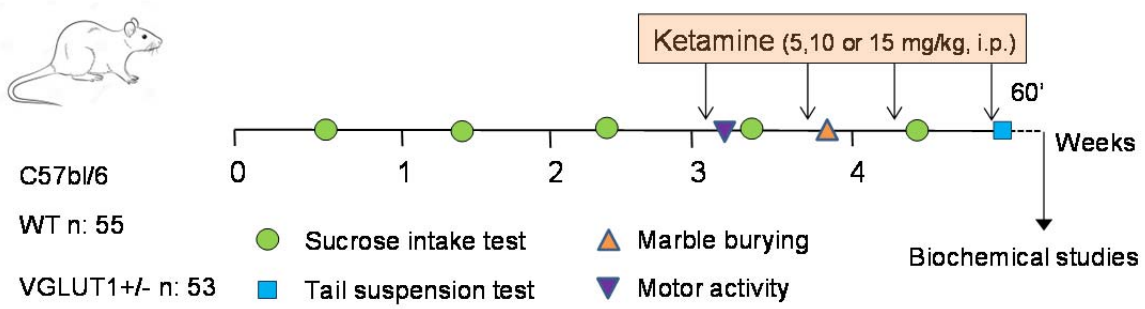

Figure 6. Experimental design followed to test the effect of effect of ketamine in the VGLUT1+/- depression model.

Experimental design 3. Effect of ketamine in the electrophysiological oscillatory activity in the frontal cortex of VGLUT1+/- mice

Here was studied how reduced glutamate input in the VGLUT1+/- mice affect the oscillatory activity of the frontal cortex. VGLUT1+/- and WT mice were implanted with deep electrodes targeting the infralimbic (IL) and frontal association area (FrA). After one week recovery, animals were connected to an acquisition system to recorded local field potentials in an open field under awake and freely moving conditions. Then, animals were treated with an acute dose of ketamine (10 mg/kg, i.p.). Simultaneous video recordings were also used to track the behavioral state of the animals. Electrophysiological activity was first visually inspected to detect the presence of abnormal activities and then processed by using spectral techniques **(Figure 7)**.

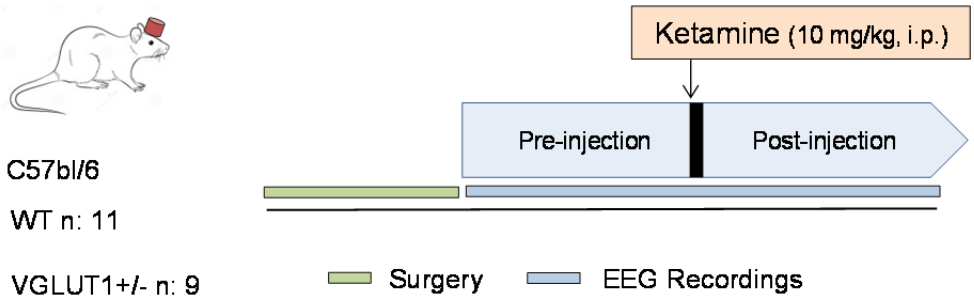

Figure 7. Experimental design followed to test electrophysiological oscillatory activity in the FrA and IL of the VGLUT1+/- and WT mice.

Experimental design 4. Behavioral effects of ketamine in the VGLUT1+/- depression model pretreated with reboxetine

This experiment was directed to rescue VGLUT1 expression levels in the PFC of VGLUT1+/- mice following a four week reboxetine (15 mg/kg, i.p.) treatment. After two weeks intake baseline for the sucrose intake solution was established, WT and VGLUT1+/- mice were subdivided into vehicle and reboxetine groups in a way that there was no difference in sucrose intake measurements between subgroups prior to the treatments. Reboxetine (15 mg/kg, i.p.) or saline were administered daily along 4 weeks. Then, all mice were washed up of drug injections for 72h. At this time point, helpless behavior in the TST was measured 60 minutes after injection of saline or ketamine (10 mg/kg, i.p.) in both WT and VGLUT1+/- mice. Following TST, tissue from the PFC was collected for biochemical studies **(Figure 8)**.

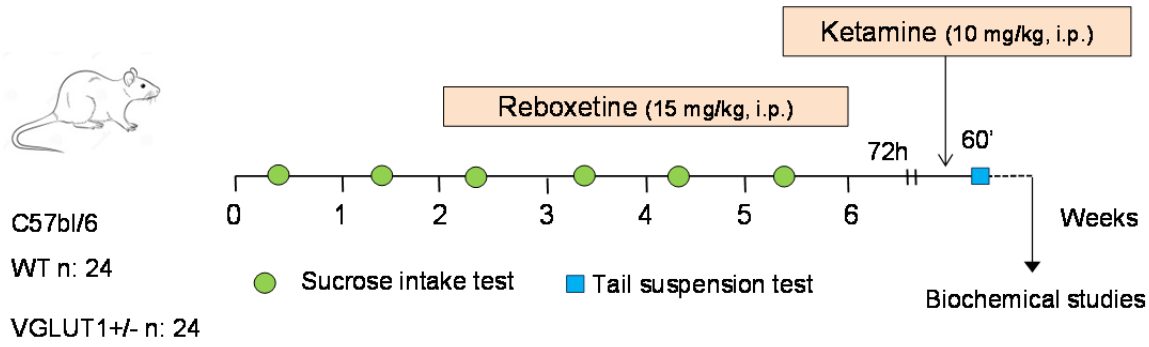

Figure 8. Experimental design followed to test the effect of ketamine in depressive-like phenotype of VGLUT1+/- mice pretreated with reboxetine.

Experimental design 5. Behavioral effects of ketamine in the VGLUT1+/- depression model with induced expression of VGLUT1 in the PFC

This experiment was directed to induce VGLUT1 protein expression using adeno-virus vectors (AAVs) that carry the VGLUT1 sequence in its WT form or a mutated glutamate transport sVGLUT1 form linked to the synapsin promoter. After three basal sucrose intake measurements, stereotaxic injections in the PFC of WT and VGLUT1+/- mice were randomly assigned to treatment with the pAAV-pSyn-VGLUT1-mCherryminisog (VGLUT1^{mCherryminisog}), the glutamate transport deficient form pAAV-pSyn-sVGLUT1-mCherryminisog (sVGLUT1^{mCherryminisog}) or the control group pAAV-pSyn-YFP (YFP) for each genotype. After one week of recovery, anhedonic behavior was evaluated with the sucrose intake test for four weeks. On the last day, TST was studied 60 minutes after ketamine injection (10 mg/kg i.p.) and then brain tissue was collected for imaging experiments **(Figure 9)**.

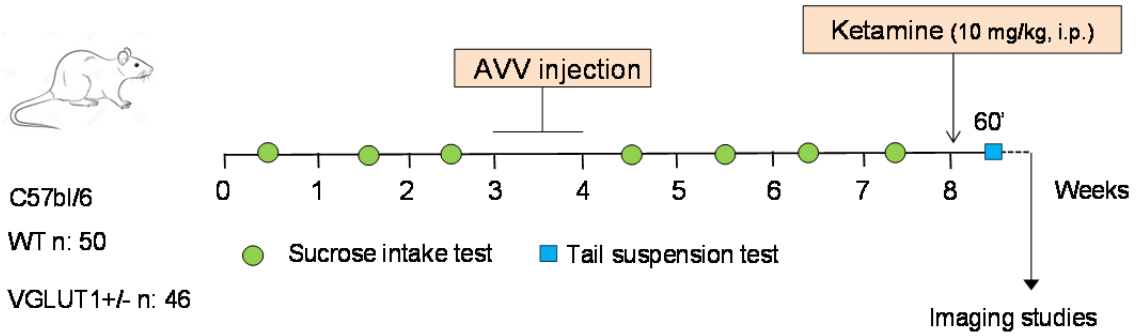

Figure 9. Experimental design followed to test the effect of ketamine in depressive-like phenotype of VGLUT1+/- mice with induced VGLUT1 expression in the PFC.

4. The VGLUT1+/- model of depression: Methods

4.1. Behavioral tests

The sucrose intake test

Anhedonic-like behavior was evaluated by monitoring of sucrose intake as described previously by (Elizalde *et al.,* 2010). Mice were first trained to drink sucrose solution by exposing them to two standard drinking bottles, one containing 2.5% of sucrose and the other tap water, for every night during one week. After this preliminary phase, mice were food deprived and exposed to the sucrose solution and water. The position of the 2 bottles (right/left) was varied randomly from trial to trial. Once a week, mice were

given a 15h exposure to the sucrose solution and tap water in their home cage as described above. Body weight measurements were taken weekly and relative sucrose intake and sucrose preference (sucrose intake/total intake) was calculated as absolute intake (g) per mouse body weight.

The tail suspension test

This test considered a model of "behavioral despair" for screening of antidepressant drugs, was carried out as originally described (Steru *et al.,* 1985). After one hour of the drug injection, mice were secured by the tail to a plastic cylinder (4 cm wide) with an adhesive tape (1 cm of the distal tail) and suspended head-down 40 cm. Mice were scored for immobility over a 6 minute test period. Immobility time was recorded as a lack of movement/struggling and motionless hanging using a video tracking system (Ethovision 11.5 XT plus multiple body point module, Noldus Information Technology B.V, Wageningen, The Netherlands).

The marble burying test

Natural burying behavior was assessed with this test. Fifteen marbles (1.5 cm diameter) were placed uniformly in a cage (45×28×20 cm) containing a constant amount of sawdust (3 cm deep). Mice were placed in the center of the cage and left for 30 minutes. The number of marbles buried was counted.

Locomotor activity

Distance travelled (cm) was measured in an open field consisting of four beige square arenas (35×35×30 cm) using a video tracking system (Ethovision 11.5 XT , Noldus Information Technology B.V, Wageningen, The Netherlands) in a softly illuminated experimental room. Mice were placed in each box and spontaneous locomotor activity was analysed in the novel cage. Distance travelled and speed was recorded during a 30 minute period.

4.2. 5-HT_{1A} agonist induced hypothermia

8-OH-DPAT induced hypothermia as described previously by (Garcia-Garcia *et al.,* 2013). Core body temperature was measured using a thermistor probe inserted 2 cm into the mice rectum in the VGLUT1+/- mice model. Mice received a single subcutaneous injection of the 5-HT_{1A} receptor agonist 8-hydroxy-N, N-dipropyl-2-aminotetralin (8-OH-DPAT) 0.25 mg/kg or vehicle (0.9% NaCl). Core body temperature

at baseline and 30, 60 and 90 min after 8-OH-DPAT injection was measured in the VGLUT1+/- model. Data are presented as the difference in core body temperature over basal values.

4.3. *In vivo* electrophysiological oscillatory activity in the brain

These studies were carried out in collaboration with the electrophysiology laboratory of Dr. Artieda and Dr. Valencia (Center for Applied Medical Research CIMA, Navarra, Spain).

Stereotactical surgery for electrode implantation

Mice were stereotactically implanted with multicontact electrodes following the atlas (Paxinos, 2001). Three electrodes were implanted unilateral in the infralimbic cortex (IL, coordinates: AP: 1.94 mm; L: 0.3 mm; DV: -1.5 mm) 200 µm of separation. In addition, one screw was placed in the frontal association area (FrA, coordinates: AP: 2.8 mm; L: 1.5 mm) and another two as ground and reference were symmetrically placed over the cerebellar region (left and right respectively). For that purpose, a 3D printed custom-made pedestal was assembled with tungsten (wolfram) electrodes 50 µm diameter (California Fine Wire) and stainless steel screws 0.9 mm diameter (PTS-UK) **(Figure 10)**.

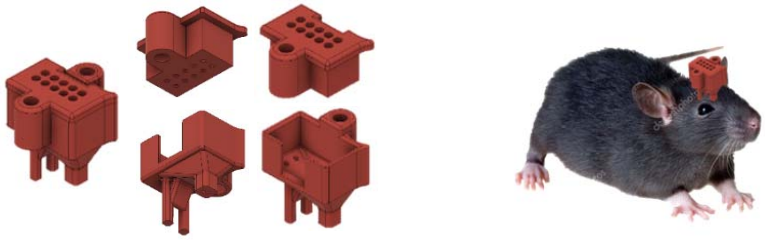

Figure 10. 3D printed custom-made pedestal model. From left to right: complete pedestal model; pieces viewed from bellow; pieces viewed from above; mounted on a mouse.

During surgery, animals were firstly anesthetized with intraperitoneal ketamine-xylazine injection: ketamine 75 mg/kg (ketamidor 100mg/ml, Dechra, UK), xylazine 10 mg/kg (rompum 2%, ELASA). Then scalp was retired with scissors and the periosteum was retired with scalpel and hydrogen peroxide (5.1%, CINFA). Next, little trepans were manually done with a small drill tip (0.7 mm diameter) and a stereotactical arm (10 micron precision frame and micromanipulator, 1430 KOPF; tip mouse ear bars, 822 KOPF) was used for implanting the custom made pedestal with each electrode and screw in the appropriate coordinates. Finally it was held with dental cement. In the post-surgery saline (Sterile saline 0.9%, Braun) was intraperitoneally injected in order

to rehydrate them from lost fluids and ketoprofen (Ketofen 1%, Merial) was intramuscularly injected as painkiller. After the surgery mice were replaced into another cage for at least one hour post-surgery until partial recovery and moved then to the quarantine room until total recovery before recordings.

Electrophysiological recordings

Animals were recorded inside an open field (33.5 cm x 44.5 cm) cage placed inside a custom-made Faraday cage shielded from external electrical fields. Mice were connected to the recording system through a head stage (RHD2132 16-Input Amplifier Board, Intan Inc.) and connected with a cable in the top of the cage (92 cm) with a rotary commutator that allowed mice to freely move during the recordings. Electrical activity was recorded with a sampling frequency of 5 kHz and stored with the Intan GUI software for later analysis. Simultaneously video recording and online tracking of the mice position performed and stored using the BONSAI software. Each recording comprised of 12 minutes of basal register and 12 minutes with ketamine (10 mg/kg, i.p.) injection (Anesketin® 100 mg/ml, Dechra, UK). Well stablished threshold of 2 cm/s to differentiate between active (>2 cm/s) and stillness (<2 cm/s) period was adopted.

Analysis of oscillatory activity

Power spectral estimates were obtained according to previous methods (Nicolás *et al.,* 2011) and separately for two different time slots: 12 minutes pre-injection and 12 minutes post-injection. We also distinguished and separated periods of locomotor activity and stillness that were delimited according to the tracking information obtained during the electrophysiological register. Then, mean ± CI (confidence interval) of the grouped spectra were plotted to obtain a qualitative description of the spectral content in the 0-250 Hz frequency range containing; delta (δ; 0-4 Hz), theta (θ; 4–8 Hz), gamma (γ; 30–100 Hz) and high-frequency oscillations (HFO; 100–200 Hz). Finally, a custom made script was used to visualize and to annotate the frequency and amplitude/intensity of the power spectrum peaks on each individual spectrum. Central frequency (Hz) and power spectral density (PSD) values were then exported for further statistical analysis.

4.4. Adeno-associated virus vectors (AAVs) expressing VGLUT1

Design of adeno-associated virus vectors expressing VGLUT1

Recombinant adeno-associated virus vectors (AAVs) were engineered with either WT or the glutamate transport deficient sVGLUT1 inserts **(Figure 11)** in collaboration with the laboratory of Dr. Etienne Herzog (University of Bordeaux, France).

Briefly, both WT and mutated VGLUT1 constructs were tagged with mCherryminisog at the C-terminus (Qi *et al.,* 2012) and the expression of both pAAV-pSyn-VGLUT1 mCherryminisog (VGLUT1^{mCherryminisog}) and pAAV-pSyn-sVGLUT1 mCherryminisog (sVGLUT1^{mCherryminisog}) inserts was controlled by the human synapsin promoter. Mutated form of VGLUT1 (sVGLUT1) consisted on the mutation of three 3 residues of arginine in three transmembrane domains (TM1, TM4 and TM7) by glutamine (R80Q), lysine (R175K) and glutamine (R314Q) respectively. Then, serotype 9 AAV particles were generated by transient transfection of HEK293T cells and viral stocks as previously described (Berger *et al.,* 2015).

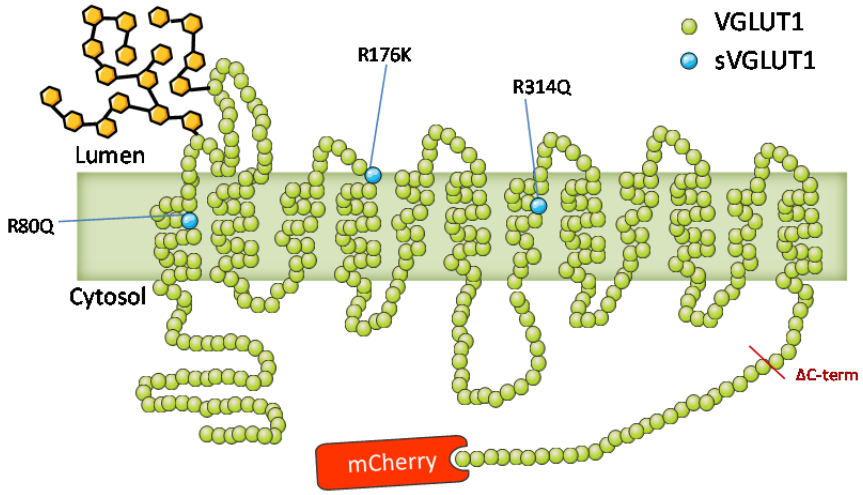

Figure 11. Structure of VGLUT1 and sVGLUT1 showing the 12 transmembrane domains and sites of the mutation. All constructs carried a mCherryminisog tag at the C-terminal domain.

Viral vectors VGLUT1^{mCherryminisog} and sVGLUT1 ^{mCherryminisog} were generated together with the control viral vector pAAV-pSyn-YFP. Viral stocks were stored in 10 µl aliquots at -80°C before use. Viral titles were estimated by quantification of the p24 capsid protein using the HIV-1 p24 antigen immunoassay (ZeptoMetrix Corporation, Buffalo, NY, USA) according to the manufacturer's instructions. All productions were between 100 and 300 ng/µl of p24.

Expression of these three AAVs was validated *in vitro* in hippocampal cell primary cultures (Zhang *et al.,* 2018). Moreover, previous *in vivo* study carried out in our laboratory had tested the optimal dose required for imaging VGLUT1^{mCherryminisog} expression (unpublished data) in both cell bodies and fibbers of the PFC as well as in fibbers of the DRN.

Stereotaxic injections of adeno-associated virus vectors expressing VGLUT1

For stereotaxic delivery of the AAV vector, adult WT and VGLUT1+/- mice were randomly assigned to treatment with the VGLUT1^{mCherryminisog}, sVGLUT1^{mCherryminisog} or control groups injected with YFP. Mice were anesthetized by i.p. injection of ketamine/xylazine (0.1/0.05 g/kg of body weight) and positioned on a stereotactic frame (Steeling, Wood Dale, USA). Mice were then injected using 0.5 µL of YFP, VGLUT1^{mCherryminisog} or sVGLUT1 ^{mCherryminisog} (1.47E12, 2.01E14 and 2.03E14 viral genomes/µL, respectively) bilaterally into the PFC (+2 mm AP, 1 mm ML, -1.7 mm DV from bregma), of adult mice based on established coordinates (Hof *et al.*, 2000). Vector delivery was performed at a rate of 200 nL/minute using a Hamilton syringe (Hamilton Company, Sarasota, FL, USA) with 33 gauges needle (World Precision Instruments) in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). After the injection, the needle remained in place for two more minutes and then carefully retracted in order to avoid vector backflow. Subsequently, the scalp was sutured and the mouse was caged individually until full recovery from anesthesia.

Imaging of adeno-associated virus vectors expressing VGLUT1

Tissue preparation. Mice were sacrificed four weeks after the stereotaxic surgery and 90 minutes after last drug injection. The animals received an anesthetic overdose of sodium pentobarbital (60 mg/mL, Merck) and were transcardially perfused with 0.9% saline followed by ice cold 4% paraformaldehyde in 0.1M phosphate buffer (pH=7.4) prior to brain extraction. For histological processing, the whole brain was removed, post-fixed in 4% paraformaldehyde (2h), cryoprotected in a 30% sucrose solution (in phosphate buffer) and stored at 4°C until they sank. Serial coronal microtome sections (40 µm) were collected throughout the PFC (Bregma 2.40 to 1.40) and were stored in cryoprotectant solution at -20°C until processing.

Serial free-floating sections (one every four 40 µm sections) of the whole PFC were used. Sections were mounted on gelatin-coated slides, air-dried in a light protected chamber. Subsequently, mounting medium (Immunomount, Thermo Scientific, Kalamazoo, MI, USA) was added and cover-slipped.

Image capture. Immunoreactivity of the rostro-caudal extents throughout the PFC (7-8 sections) of transgene expression of all animals used in behavioral experiments was assessed by the fluorophore YFP as control or mCherryminisog as target. Fluorescence of YFP, VGLUT1^{mCherryminisog} and sVGLUT1^{mCherryminisog} were assessed using a confocal fluorescence microscope (LSM 510 META, Carl Zeiss, Jena, Germany) or a conventional epifluorescence microscope (Eclipse Ni, Nikon Instruments Europe BV, Amsterdam, Netherland). The amount of VGLUT1^{mCherryminisog} expression or sVGLUT1^{mCherryminisog} was detected by Western blot using the antibody with specificity to VGLUT1^{mCherryminisog}. To confirm the expression of the VGLUT1^{mCherryminisog} a western-blot using an antibody that recognized both endogenous VGLUT1 and exogenous VGLUT1^{mCherryminisog} (VGLUT1L-53P3) was used (Herzog *et al.,* 2011).

Image quantification. Detection and quantification of mCherryminisog or YFP fluorescent signal in brain sample images was carried out using a plugin developed for Fiji/ImageJ, an open-source Java-based image processing software (Schneider *et al.*, 2012). The plugin was developed by the Imaging Platform of the Center for Applied Medical Research (CIMA). First of all, a region of interest (ROI) is manually delineated in each of the brain hemispheres. A background subtraction is then carried out using the “Rolling Ball Background Subtraction”, plugin developed for Fiji by Michael Castle and Janice Keller, from the Mental Health Research Institute of the University of Michigan, which was originally based on the work of Sternberg (Sternberg, 1983). Positive fluorescent signal in each ROI is then retrieved by thresholding the resulting image. A global histogram-derived thresholding method is applied for this purpose, specifically Otsu's clustering algorithm that searches for the threshold that minimizes the intra-class variance (Otsu, 1979). Finally, the relative fluorescence signal area in each hemisphere ROI is quantified from the final segmentation.

4.5. Western blot studies

Tissue preparation. After the last i.p. injection animals were sacrificed by cervical dislocation and their brains were rapidly removed and dissected in an acrylic mouse brain slicer matrix with 1.0 mm coronal slice intervals (Zivic Instruments, Pittsburgh, PA, USA). Using a mouse brain atlas (Hof *et al.*, 2000), a 1mm slice was taken from the cingulate cortex including the prelimbic (PL) and infralimbic (IL) section of the PFC (bregma 2.20 mm through bregma 1.20 mm) and dissected out bilaterally using a scalpel and kept at -80°C. In addition, the brain stem containing the DRN was dissected. Then, western blot studies were carried out directed to study the expression of different synaptic plasticity and glutamate targets.

Western blot analysis was carried out in PFC tissues collected from mice killed 90 minutes after the last drug injection. PFC and DRN dissected from the mice were sonicated in a cold lysis buffer with protease inhibitors (0.2M NaCl, 0.1M HEPES, 10% glycerol, 200mM NaF, 2mM Na4P2O7, 5mM EDTA, 1mM EGTA, 2mM DTT, 0.5mM PMSF, 1mM Na3VO4, 1mM benzamidine, 10 mg/mL leupeptin, 400 U/mL aprotinin). The homogenate was centrifuged at 14000 g at 4ºC for 20 minutes and the supernatant aliquoted and stored at -80ºC. Protein concentration was determined by Bradford (BIO-RAD, Hercules, CA, USA). For western studies directed to the detection of induced expression of VGLUT1^{mCherryminisog} the synaptosome fraction (P2) was prepared following the protocol of (Peyrusse *et al.,* 2018).

Electrophoresis. Equal amounts of protein (20 μg per lane) were loaded of each sample after being mixed with equal volume of loading buffer (0.16M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue, 0.1M DTT). Then, proteins were separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel (6-10%) under reducing conditions and transferred onto a nitrocellulose membrane (10x7 cm) (Hybond-ECL; Amersham Bioscience). The trans-blots were blocked for 1h with Odyssey® Blocking Buffer (PBS) (LICOR®, Biosciences, and Lincoln, NE, USA). Subsequently, membranes were incubated overnight at 4ºC with primary antibodies (1:1000 dilution) detailed in **(Table 1)**. β-Actin (1:10000, Sigma-Alrich, St. Louis, MO, USA) was used as internal control if not specified. Moreover, Odyssey® goat anti-rabbit and anti-mouse secondary antibodies (LI-COR®, Biosciences, Lincoln, NE, USA) were diluted to 1:5000 in TBS with 5% BSA and then incubated for 1 hour in room temperature.

Table 1. List of primary antibodies used for Western blot studies.

Antibody	Host	Reference
Akt	Rabbit	9272S, Cell Signaling Technology
eEF2	Rabbit	2332S, Cell Signaling Technology
GluA1	Rabbit	Ab1504, Merck Millipore
pro-BDNF	Rabbit	Ab72439, Abcam
p-Akt	Rabbit	9271S, Cell Signaling Technology
p-eEF2	Rabbit	2331S, Cell Signaling Technology
p-ERK	Rabbit	9106S, Cell Signaling Technology
p-GluA1	Rabbit	04-823, Merck Millipore
p-mTOR	Rabbit	2971S, Cell Signaling Technology
p-P70 S6k	Mouse	9204S, Cell Signaling Technology
p-S6	Rabbit	4858S, Cell Signaling Technology
VGLUT1	Rabbit	Donated Dr. S. El Mestikawy (Herzog *et al.,* 2001)
VGLUT1L-53P3	Rabbit	Donated Dr. Herzog (Herzog *et al.,* 2011)
VGLUT2	Mouse	Donated Dr. S. El Mestikawy (Herzog *et al.,* 2001)
β-Actin	Mouse	A1978, Sigma Alrich

Finally, bands were visualized using Odyssey® Infrared Imaging System and quantified with Image Studio Lite 5.2 (LI-COR® Biosciences, Lincoln, NE, USA). Results were calculated as the percentage of optical density values of the control saline mice.

5. The MT_2 receptor knock-out model of depression: Experimental designs

Experimental design 1. Depressive-like phenotype and circadian temperature disruption of the MT_2-/- model

Here we investigated the role of the melatonergic receptor subtypes MT_1 and MT_2 in the regulation of anhedonia together with other symptoms of depression as well as in circadian temperature. Anipill® pills that allow body temperature recordings were implanted into the peritoneum of MT_1-/-, MT_2-/- and WT mice. Following two weeks recovery, sucrose intake test was applied once a week on three consecutive weeks in order to measure anhedonic behavior. On the third week, novelty seeking behavior was tested by the conditioned-place preference (CPP) test. Following CPP, the hypothermic response of 8-OH-DPAT was tested as a measure of 5-HT_{1A} autoreceptor sensitivity. Throughout all these weeks, CBT was obtained every 5 min **(Figure 12).**

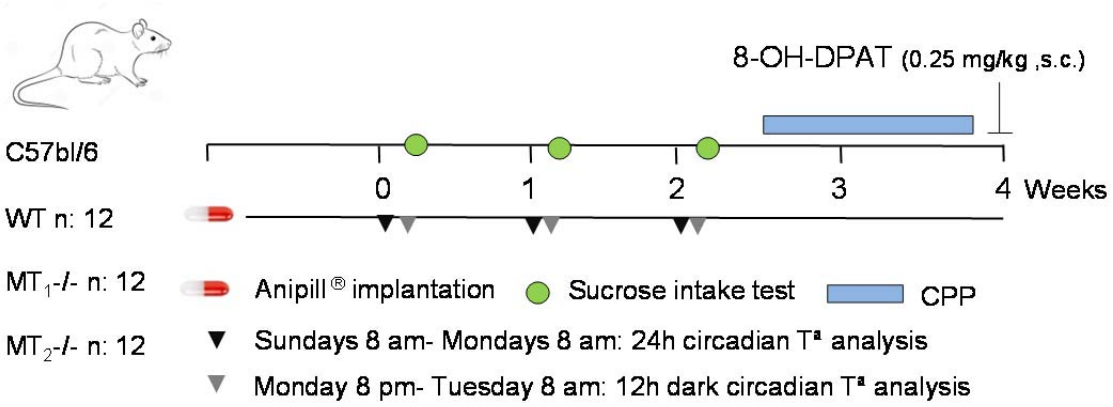

Figure 12. Experimental design followed to test the behavioral phenotype and circadian temperature of MT_1-/- , MT_2-/- mice and WT littermates.

Experimental design 2. Stress vulnerability of the MT_2-/- depression model

MT_1-/-, MT_2-/- and WT mice were exposed to CSDS for ten days and approach-avoidance behavior was analyzed in the social interaction test one month after **(Figure 13)**.

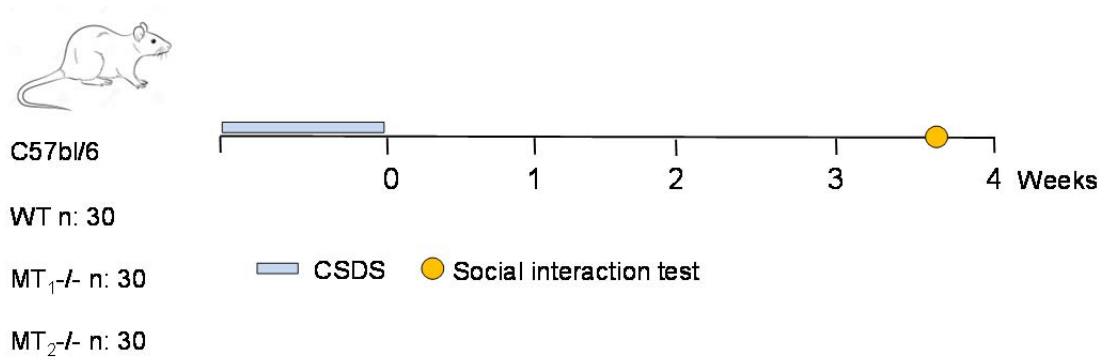

Figure 13. Time schedule followed to test the stress vulnerability of MT_1-/-, MT_2-/- and WT littermates to develop social avoidance following CSDS exposure.

Experimental design 3. Effect of the antidepressant reboxetine on depressive-like behavior and circadian CBT in the MT_2-/- depression model

Anipill® pills were implanted into WT and MT_2-/- mice. Following recovery, mice were daily treated (except Sundays) with saline or reboxetine (15 mg/kg, i.p.) for four weeks. Anhedonia, novelty-seeking behaviour and 5-HT_{1A} autoreceptor sensitivity were tested as in experiment 1. Moreover, helplessness by using the tail suspension test (TST) was evaluated on the 4th week of treatment **(Figure 14)**.

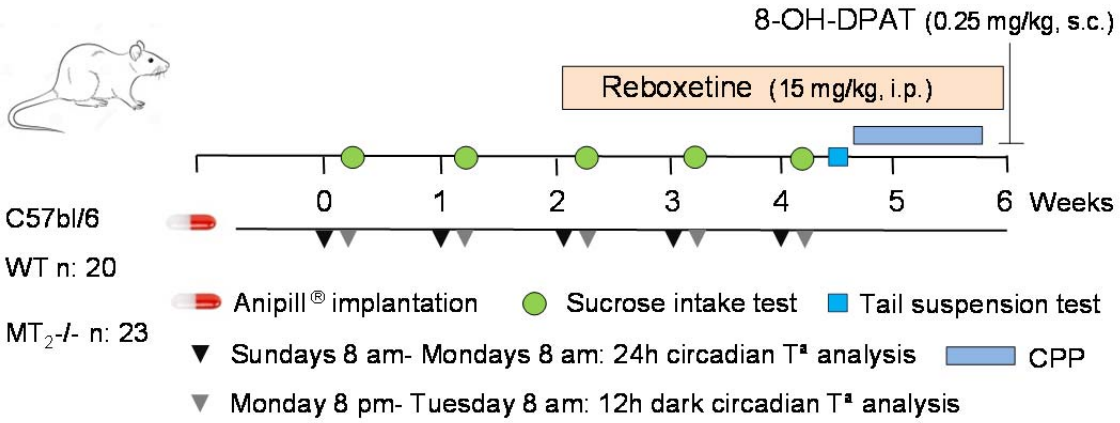

Figure 14. Experimental design to test the effect of the classic antidepressant reboxetine on the depressive-like behavior and disrupted circadian temperature of the MT_2-/- model.

Experimental design 4: Effect of the rapid acting antidepressant ketamine on depressive-like behavior in the MT_2-/- depression model

WT and MT_2-/- mice were treated with saline or ketamine (15 mg/kg, i.p) and during this time, anhedonia and helplessness (TST) were evaluated **(Figure 15)**.

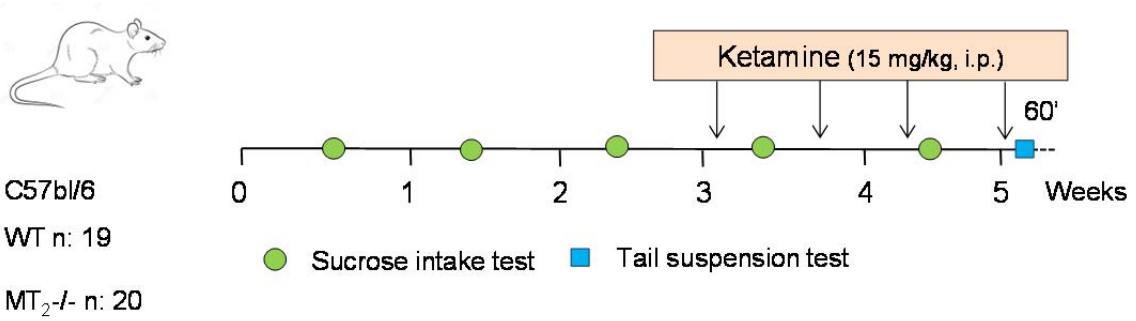

Figure 15. Experimental design followed to test the effect of ketamine on the depressive-like behavior of the MT_2-/- model.

6. The MT_2 receptor knock-out model of depression: Methods

6.1 Behavioral tests

The sucrose intake test

Anhedonic-like behavior was evaluated by monitoring of sucrose intake as described by (Elizalde *et al.,* 2010) as explained in the VGLUT1+/- model of depression: Methods.

The tail suspension test

This test considered a model of "behavioral despair" for screening of antidepressant drugs, was carried out as originally described (Steru *et al.,* 1985) as explained in the VGLUT1+/- model of depression: Methods.

Core body temperature measurement

Anipill® pills (BodyCap-medical, France) were used for a weekly monitoring of circadian CBT. For the first step of the procedure, mice underwent an intraperitoneal implantation of one capsule under general anesthesia with pentobarbital (40 mg/kg. i.p.). An incision of 1-2 mm in length was performed and the capsule was placed in between the peritoneal space. After implantation, each mouse received a dose of buprenorphine (0.1 mg/kg, s.c.) for two days in order to decrease nociception due to the laparotomy.

The data sampling frequency of the sensor was selected to 5 (validation experiment) or 15 minutes (Reboxetine treatment). Data was transmitted by a telemetric technology to a monitor and twenty-four hours temperature profiles were generated for each animal in standard conditions (free access to water and food) on the quietest day for the animal facility (Sundays 8 a.m. to Mondays 8 a.m.). In order to avoid manipulation interferences none of the treatments were performed on Sundays. The analysis of temperatures was made by analyzing the area under the curve (AUC) generated in the different temperatures recorded on every Sunday. Because none of the curves reached below 34ºC, the areas below this temperature were subtracted. Circadian dark period curve was obtained in basal conditions (Sundays 8 p.m. to Monday's 8 a.m.) and during the sucrose intake test (Mondays 8 p.m. to Tuesdays 8 a.m.) in order to study mice sensitivity to reward exposure. GraphPad Prism 6.01 software (GraphPad, La Jolla, CA, USA) was used in order to apply a local regression method (robust LOESS) to smooth the data represented in order to remove noise-like features and emphasize significant trends.

The novel object-induced conditioned place preference (CPP) test

Novel object-induced CPP test was applied with minor modifications (Douglas *et al.,* 2003). A conventional conditioned place preference apparatus was used. The apparatus had two chambers separated by a central narrow compartment. Each chamber had an inside dimensions (25×25×20 cm) and the central compartment had a flat grey floor and grey walls (8.5×25×20 cm). This center compartment is connected with the other two via guillotine doorways (4x4 cm). The two compartments differ in wall striping (vertical versus horizontal alternating grey and white lines, 1.5 cm in width). At the start of preference testing, each mouse was placed in the center compartment.

Briefly, on days 1 and 2 of the experiment, each mouse was placed alone on a central compartment (C) and the animal was allowed to move freely throughout the two chambers (A or B) for 15 minutes. The amount of time in each compartment during the days 1 and 2 was used as the baseline. For each mouse the preferred compartment was identified. The non-preferred compartment was defined as the compartment in which mice spent less time.

At unpaired-days (3, 5, 7 and 9) of the conditioning trial, each mouse was confined to the non-preferred compartment for 10 minutes. The mouse was then returned to the cage and 1 hour later it was confined to the other compartment for 10 minutes. At paired-days (4, 6 and 8) each mouse was confined first to the preferred compartment for 10 minutes and then 1 hour later confined to the non-preferred compartment for 10 minutes. All mice had access to an object only in the non-preferred compartment and a different object was used each day. Specifically Lego bricks objects were used. On day 10, mice were placed in the central compartment and then allowed free access to both compartments for 15 minutes. The percentage of time spent in each compartment was calculated using the video tracking system (Ethovision 11.5 XT, Noldus Information Technology B.V, Wageningen, The Netherlands) **(Figure 16)**.

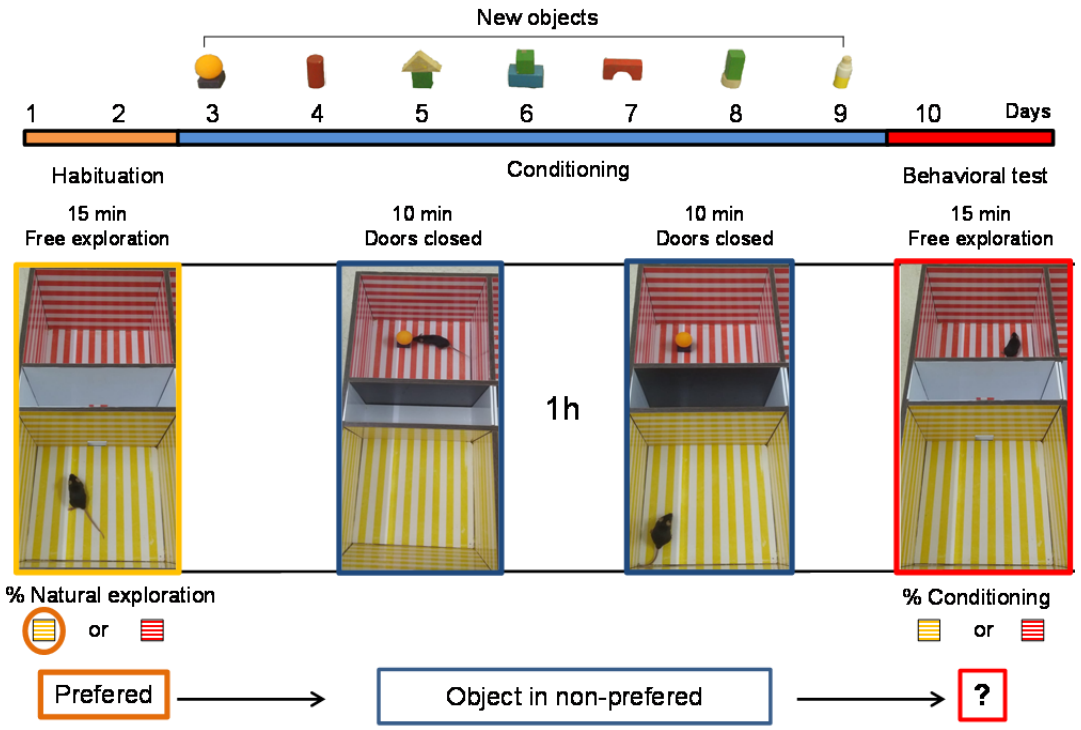

Figure 16. Graphic representation of the novel object-induced conditioned place preference (CPP) protocol.

Chronic social defeat stress procedure (CSDS)

CSDS procedure was carried out using a similar method described by (Venzala et al. 2012). Briefly, mice were submitted to social defeat stress for ten consecutive days. Every day, each mouse was introduced into the home cage of an unfamiliar resident. Resident mice were CD1 retired breeders selected for their attack latencies reliably shorter than 30s upon three consecutive screening tests. Once the experimental

mouse had been physically defeated by three attacks, both animals (defeated and aggressor) were maintained in sensory contact for 24h using a metallic mesh dividing the resident home cage in two halves. During these ten days control mice were individually housed in equivalent cages but with members of the same strain in the opposite half. Subsequently, mice were placed in individual cages and social interaction test was performed one month after.

Social interaction test

Social interaction test measures the approach-avoidance behaviour towards an unfamiliar social target. Mice were introduced into an open field and trajectory was recorded in two 2.5 min sessions. During the first session ("no target") an empty metallic mesh cage was located at one end of the field and in the second one ("target"), an unfamiliar mouse from the same strain was introduced into the mesh cage. Between sessions, mice were placed back into their home cage for approximately 1 min. Difference in the time spent in the "interaction zone" (7-cm wide corridor surrounding the metallic cage) between sessions was calculated.

6.2 5-HT_{1A} agonist induced hypothermia

8-OH-DPAT induced hypothermia as described by (Garcia-Garcia *et al.,* 2013). Core body temperature was measured using a thermistor probe inserted 2 cm into the mice by Anipill® pill recording. Mice received a single subcutaneous injection of the 5-HT_{1A} receptor agonist (0.25 mg/kg) or saline. Core body temperature was measured at 5 minute intervals for up to 90 minutes. Data are presented as the difference in core body temperature over basal values.

7. Statistical analysis

The VGLUT1+/- model

The effect of genotype (VGLUT1+/- and WT) on sucrose intake, immobility time and VGLUT1 protein expression along the different independent experiments were analysed by Two-Way ANOVA. The 95% calculate a confidence interval (CI) for each population was calculated. In addition, Hartigans´ dip test for unimodality was applied.

The effect of genotype and drug or AAV treatment on sucrose consumption and on temperature differences induced by 8-OH-DPAT along time were analysed by Two-

Way ANOVA, repeated measures. Each time point was analysed by Two-Way ANOVA followed by Tukey *post-hoc* test when significant interaction.

The effect of genotype and drug or AAV treatment on different behavioural paradigms (immobility time, marbles buried, distance travelled) as well as expression levels of different proteins were analysed by Two-Way ANOVA followed by Tukey *post-hoc* test when significant interaction.

The effect of genotype and ketamine treatment on power spectral density components (intensity and frequency of oscillatory activity) for each of the identified bands (δ, θ, γ and HFO) were analysed by Two-Way ANOVA followed by Sidak *post-hoc* test when significant interaction.

The MT_2-/- model

In the melatonin (MT) receptor deficient models, the effect of genotype (MT_1-/-, MT_2-/- and WT) on sucrose consumption, preference for the conditioned compartment and AUC defined by CBT were analyzed using One-way ANOVA followed by Tukey *post-hoc* test. In addition, for MT_2-/- and WT littermates, mean temperature differences between the nocturnal CBT of a rewarding night and a standard night were analysed by Student t test.

The effect of genotype (MT_1-/-, MT_2-/- and WT) on temperature differences induced by 8-OH-DPAT along time were analysed by Two-Way ANOVA, repeated measures. Each time point was analysed by Two-Way ANOVA followed by Tukey *post-hoc* test.

The effect of genotype (MT_1-/-, MT_2-/- and WT) and CSDS on time spent in the interaction zone in the social interaction test was analysed by Two-Way ANOVA followed by Tukey *post-hoc* test.

The effect of genotype (MT_2-/- and WT) and drug treatment on sucrose consumption and on temperature differences induced by 8-OH-DPAT along time were analysed by Two-Way ANOVA, repeated measures. Each time point was analysed by Two-Way ANOVA followed by Tukey *post-hoc* test when significant interaction.

The effect of genotype (MT_2-/- and WT) and drug treatment on different behavioural paradigms (immobility time, preference for conditioned compartment), AUC defined by CBT as well as mean temperature differences between the nocturnal CBT of a rewarding night and a standard night were analysed by Two-Way ANOVA.

Data analyses were performed using GraphPad Prism 6.01 software (GraphPad, La Jolla, CA, USA) and SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA). All videorecordings were analysed by Ethovision 11.5 XT, (Noldus Information Technology, Wageningen, Netherlands).

Results

The VGLUT1+/- genetic model of depression

1. The VGLUT1+/- genetic model of depression: behavioral, molecular features and antidepressant action

1.1. Depressive-like phenotype of the VGLUT1+/- genetic model

We have investigated here how reduced VGLUT1 transporter levels influence depressive-like behavior. A wide population of VGLUT1+/- and WT littermates from several independent experiments were grouped for statistical analysis.

Sucrose intake test. Relative sucrose intake (g/body weight) corresponding to WT (n=245 in total) and VGLUT1+/- mice (n=236 in total) from 14 independent experiments was studied. VGLUT1+/- mice showed markedly lower sucrose intake ($p<0.0001$) than WT littermates, indicative of anhedonic-like behavior **(Figure 17A)**. 95% Confidence interval revealed a systematic difference between WT and VGLUT1+/- mice regarding sucrose intake. There is evidence of unimodality in both genotypes ($p=0.85$ and 0.87 respectively).

Tail suspension test. Immobility time (s) of VGLUT1+/- mice (n=49 in total) was compared to WT littermates (n=63 in total) from 6 independent experiments. VGLUT1+/- mice showed a significant higher immobility time ($p<0.05$) compared to WT, indicative of helpless behavior (**Figure 17B)**. Statistical analysis revealed that both WT and VGLUT1 mice follow a normal distribution regarding immobility time.

VGLUT1 expression levels. VGLUT1 expression in protein extracts from the PFC of VGLUT1+/- mice (n=34) and WT littermates (n=35) from 5 independent experiments was compared. VGLUT1 PFC levels of VGLUT1+/- mice was 58.7±3.1% of WT ($p<0.0001$) **(Figure 17C)**.

Correlation studies. In this model, VGLUT1 levels from both genotypes and anhedonic behavior measured by sucrose intake showed a significant correlation ($r=0.67$, $p<0.0001$) **(Figure 17D)**. In addition, splited by genotype WT ($r=0.59$, $p<0.05$) and VGLUT1 levels in the heterozygous correlated ($r=0.46$, $p<0.01$) with anhedonic behavior. Yet, no correlation was found between VGLUT1 levels and helpless behavior (data not shown).

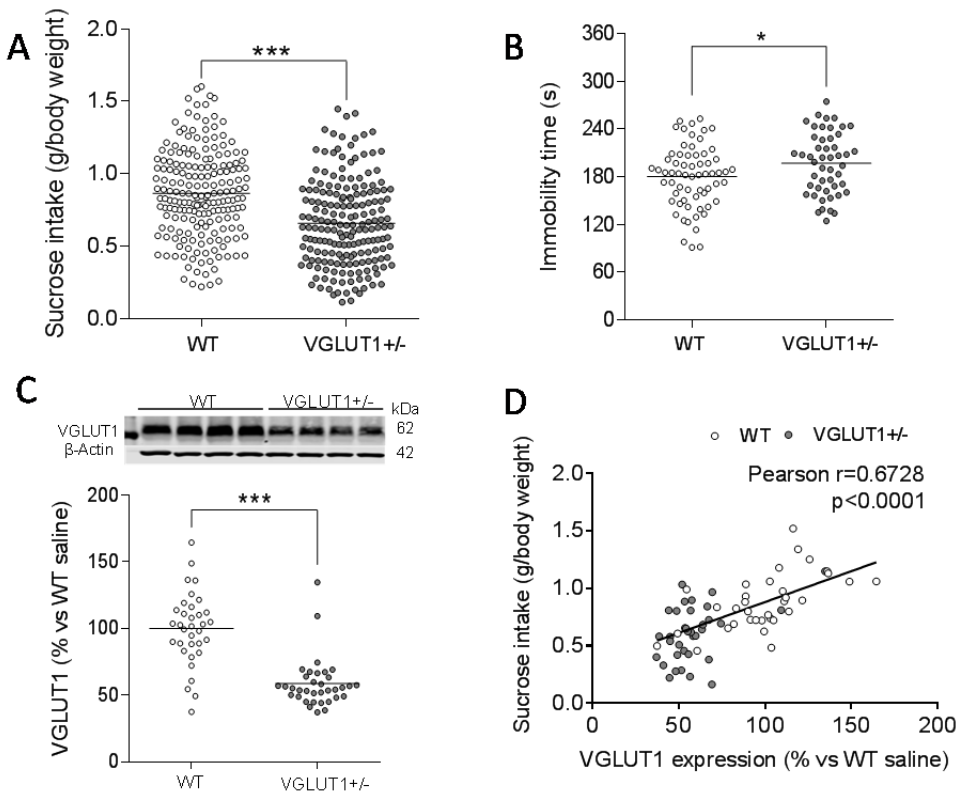

Figure 17. Depressive-like phenotype of the VGLUT1+/- genetic model. **(A)** Anhedonic behavior in the sucrose intake test ($F_{1.453}$=49.7 p<0.0001), **(B)** helpless behavior in the TST ($F_{1.100}$=6.6 p=0.015), and **(C)** VGLUT1 expression levels in the PFC of VGLUT1+/- and WT littermates ($F_{1.59}$=51.2 p<0.0001). **(D)** Correlation between VGLUT1 levels and anhedonia for both genotypes. Data show mean ± SEM of (A) sucrose intake (g/body weight), n=233-245 mice/group, (B) immobility time (s), n=49-63 mice/group and (C) optical density values vs control, n=34-35 mice/group.***p<0.0001, *p<0.05, main effects (Two-Way ANOVA).

1.2. Behavioral and molecular effects of the antidepressant reboxetine in the VGLUT1+/- depression model

Behavioral effects of reboxetine in the VGLUT1+/- depression model

Sucrose intake test. Anhedonic behavior was analyzed by weekly monitoring of the sucrose intake test. As previously observed, VGLUT1+/- mice showed lower sucrose intake (p<0.05) compared to WT littermates. In addition, reboxetine (15 mg/kg, i.p.), following two weeks of daily treatment, showed an increase in sucrose intake (p<0.05) in both genotypes **(Figure 18A)**.

Tail suspension test. Following three weeks of reboxetine treatment, and 60 minutes after injection, a clear anti-immobility action of this drug was revealed (p<0.001) in both genotypes **(Figure 18B)**. In addition, following 24h of drug washout period reexposure

to the TST induced a higher helpless behavior in VGLUT1+/- mice (p<0.05) compared to WT mice. Yet, no effect of treatment was observed anymore **(Figure 18C)**.

Marble burying test. Reboxetine reduced (p<0.0001) the number of marbles buried 60 minutes after drug injection in a 30 minute test in VGLUT1+/- mice and WT littermates **(Figure 18D)**. Yet, no effect of genotype was found in marble burying behavior.

Locomotor activity. The effect of reboxetine in locomotor activity was studied. VGLUT1+/- did not show alterations in distance travelled. However, reboxetine treatment reduced significantly (p<0.0001) the distance travelled in both genotypes, recorded for 30 minutes 1h after drug injection **(Figure 18E)**.

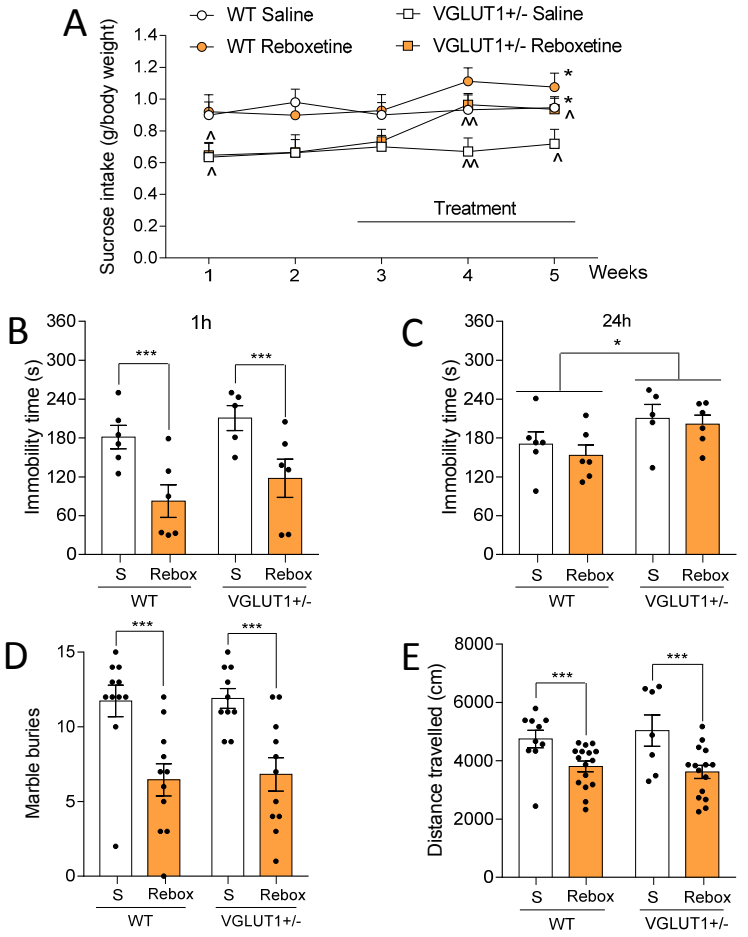

Figure 18. Behavioural effects of reboxetine in the VGLUT1+/- depression model. (A) Anhedonic behavior ($F_{1.23}$=4.48 p=0.04, treatment $F_{1.23}$=4.84 p=0.04, genotype in week 5), **(B and C)** helpless behaviour ($F_{1.19}$=16.00 p=0.0008), **(D)** marble burying ($F_{1.39}$=26.25 p=0.0001) and **(E)** locomotor activity ($F_{1.44}$=16.93 p=0.0002). Reboxetine was given at 15 mg/kg once daily. Data show mean ± SEM of (A) sucrose intake (g/body weight), n=14-15 mice/group (B and C) immobility time (s), n=6 mice/group (D) number of marbles buried, n=11 mice/group and (E) distance travelled (cm), n=12-13 mice/group. In (A) *p<0.05; main effect of treatment, ^p<0.05 main effect of genotype. In (B-E) ***p<0.0001, *p<0.05, significant main effects as indicated (Two Way ANOVA).

Effect of reboxetine on the hypothermic response mediated by a 5-HT$_{1A}$ agonist in the VGLUT1+/- depression model

Hypothermia induced by the 5-HT$_{1A}$ agonist 8-OH-DPAT was studied in WT and VGLUT1+/- mice as an *in vivo* measurement of presynaptic 5-HT$_{1A}$ autoreceptor function in the raphe nucleus (Bill *et al*., 1991). Baseline temperature values were similar in both WT (36.4±0.4ºC) and VGLUT1+/- (36.5±0.6ºC) mice. Thirty minutes after 8-OH-DPAT administration, main effects of both genotype and treatment ($p<0.05$) were observed. 8-OH-DPAT injection induced a lower hypothermic effect in VGLUT1+/- mice compared to WT littermates. Moreover, chronic reboxetine treatment enhanced this effect in both genotypes. After sixty minutes, a significant interaction ($p<0.05$) between genotype (VGLUT1+/- *vs* WT) and treatment (reboxetine *vs* saline) was observed. Particularly, while 8-OH-DPAT injection induced a lower decrease of body temperature (-0.54±0.21ºC) in VGLUT1+/- mice compared to WT mice (-1.56±0.18ºC), in VGLUT1+/- reboxetine treated mice, 8-OH-DPAT induced a decrease in body temperature (-1.27±0.22ºC) similar to WT littermates (-1.36±0.20ºC) **(Figure 19)**.

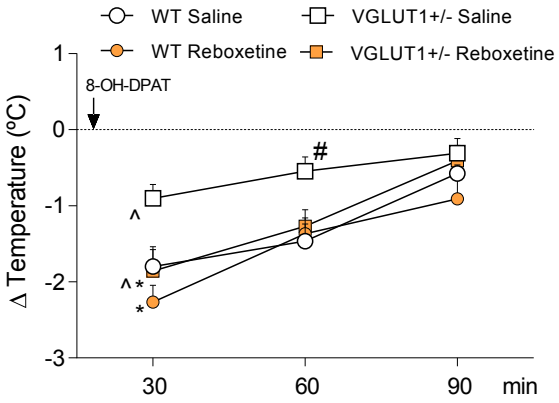

Figure 19. Effect of reboxetine on the hypothermic response to 8-OH-DPAT in VGLUT1+/- and WT mice. Hypothermic effect of 8-OH-DPAT (0.25 mg/kg, s.c.) at different time points. Data show mean ± SEM n=10-12 mice/group of body temperature. At 30 min, ($F_{1.43}$=7.1 p=0.01, genotype $F_{1.43}$=8.4 p=0.01 p=0.006 main, treatment) and at 60 min, ($F_{1.43}$=3.8 p=0.05, significant interaction). *p<0.05 main effect of treatment, ^p<0.05; main effect of genotype (two Way ANOVA), # p<0.05 vs VGLUT1+/- reboxetine group (Two Way ANOVA followed by Tukey *post-hoc* test).

Molecular effects of reboxetine in the VGLUT1+/- depression model

We next studied the effect of the antidepressant reboxetine (15 mg/kg, i.p.) in the expression of several proteins linked to glutamate function. Of these, VGLUT1-2, the phosphorylation state of GluA1 AMPA receptor subunit, the synaptic plasticity marker pro-BDNF and PI3K/Akt/mTOR intracellular signaling pathway was studied by western-

blot. Fresh tissue was collected following three weeks of antidepressant treatment and 90 minutes after the last drug injection.

Reboxetine regulation of VGLUT1, VGLUT2 and GluA1 in the VGLUT1+/- model.

As previously observed, VGLUT1 protein levels in the PFC and in the brainstem containing DRN of heterozygous were ~59% and 57% compared to WT ($p<0.01$). Chronic reboxetine increased ($p<0.01$) VGLUT1 in both genotypes and importantly rescued VGLUT1 levels in both brain areas of the VGLUT1+/- mice **(Figure 20A and B)**. Noteworthly, the expression of VGLUT2 and GluA1 were unaffected in the VGLUT1+/- model. Moreover, reboxetine increased VGLUT2 protein expression ($p<0.05$) in the mouse PFC compared to vehicle treated mice **(Figure 20C)**. Also, the phosphorylation fraction of GluA1 was significant increase compared to total levels in both genotypes ($p<0.05$) following chronic treatment **(Figure 20D)**.

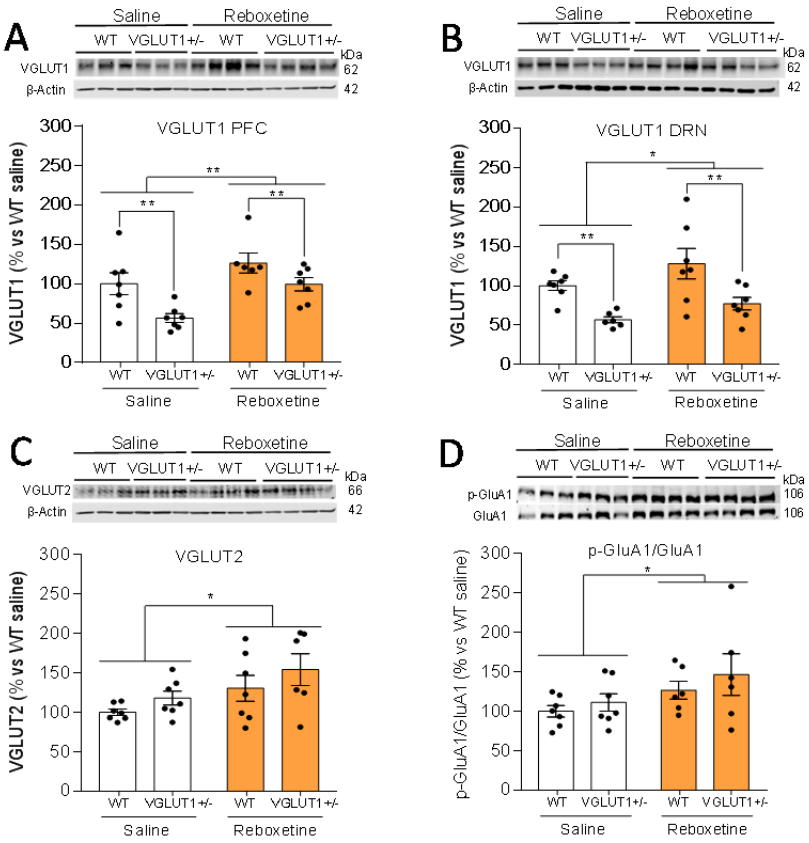

Figure 20. Reboxetine regulation of glutamate targets. (A) VGLUT1 expression levels in the PFC ($F_{1.23}$=10.71 p=0.003, treatment $F_{1.23}$=11.13 p=0.003, genotype). **(B)** Midbrain containing the DRN ($F_{1.23}$=4.43 p=0.04, treatment $F_{1.23}$=16.71 p=0.0005, genotype). **(C)** VGLUT2 ($F_{1.23}$=6.223 p=0.02) and **(D)** p-GluA1/GluA1 expression levels in the PFC ($F_{1.22}$=4.258 p=0.05). Data show mean ± SEM n=6-7 mice/group for all proteins. **p<0.01, *p<0.05, significant main effects (Two Way ANOVA).

Reboxetine regulation of synaptic plasticity marker pro-BDNF in the VGLUT1+/- model.
Subsequently, it was checked whether chronic antidepressant treatment affect the expression the synaptic plasticity marker pro-BDNF. Pro-BDNF expression in the PFC was increased by chronic reboxetine (p<0.01) **(Figure 21)**.

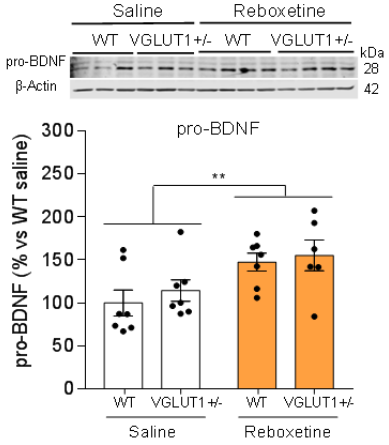

Figure 21. Reboxetine regulation of pro-BDNF in the VGLUT1+/- model. PFC pro-BDNF expression levels in VGLUT1+/- and WT littermates after chronic treatment with reboxetine $F_{1.23}$=9.947 p=0.0044). Data show mean ± SEM n=6-7 mice/group. **p<0.01; main effect of treatment (Two Way ANOVA).

Reboxetine regulation of proteins involved in the PI3K/Akt/mTOR intracellular signaling pathway in the VGLUT1+/- model.
Further, we studied the effect of the antidepressant treatment in synaptic and intracellular markers linked to the cell cycle regulator mTOR pathway. Firstly, while no effects of genotype were found, reboxetine increased p-Akt in both genotypes (p<0.05) **(Figure 22A)**. No significant changes were found in p-ERK protein **(Figure 22B)**. Moreover, reboxetine increased significantly the phosphorylation states of mTOR (p-mTOR) (p<0.05) **(Figure 22C)**, P70S6k (p-P70S6k) (p<0.01) **(Figure 22D)** and of the ribosomal S6 (p-S6) (p<0.01) **(Figure 22E)**. However, no changes were shown in the phosphorylation of the eukaryotic elongation factor eEF2 protein levels **(Figure 22F)**.

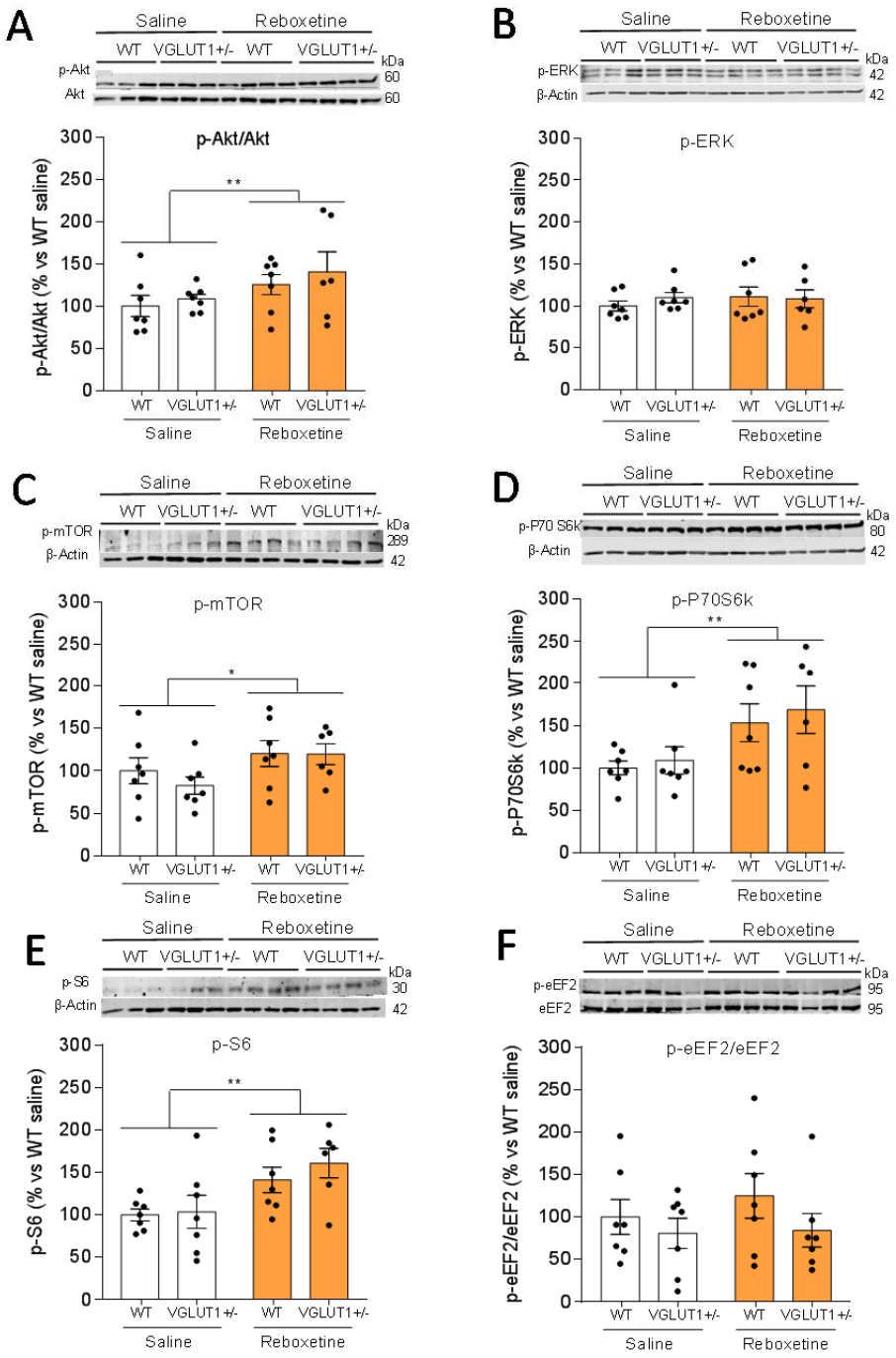

Figure 22. **Reboxetine regulation of proteins involved in the PI3K/Akt/mTOR intracellular signaling pathway in the VGLUT1+/- model. (A)** p-Akt/Akt ($F_{1.23}$=4.114 p=0.05), **(B)** p-ERK, **(C)** p-mTOR ($F_{1.23}$=4.41 p=0.046), **(D)** p-P70S6k ($F_{1.23}$=10.27 p= 0.004), **(E)** p-S6 ($F_{1.23}$=8.51 p= 0.008) and **(F)** p-eEF2/eEF2 expression levels after chronic reboxetine treatment. Data show mean ± SEM, n=6-7 mice/group for all proteins. **p<0.01, *p<0.05; main effect of treatment (Two Way ANOVA).

2. The VGLUT1+/- depression model is resistant to the rapid antidepressant action of ketamine

2.1 Behavioural effects of ketamine in the VGLUT1+/- depression model

Sucrose intake test. Anhedonic behavior was evaluated by weakly measure of the sucrose intake test. VGLUT1+/ mice showed lower levels of sucrose intake ($p<0.05$) compared to WT in the three independent experiments. Following ketamine (5, 10 or 15 mg/kg, i.p.) no effect in sucrose intake either in WT or VGLUT1+/- mice was observed **(Figure 23A, B and C)**.

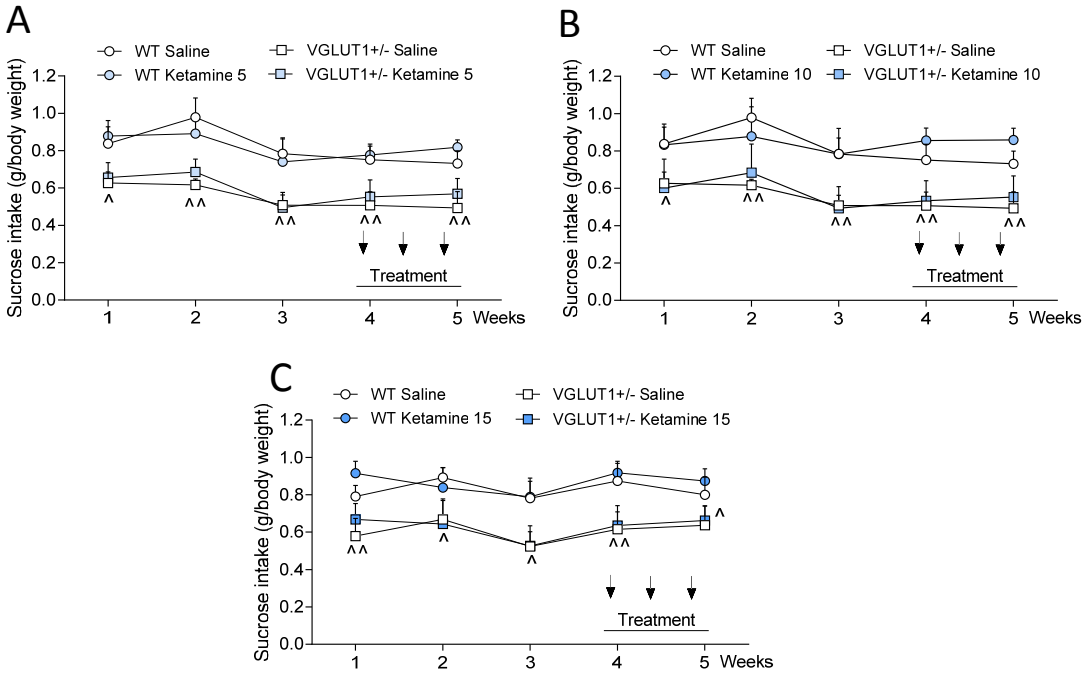

Figure 23. **Effect of ketamine in the anhedonic-like behavior of VGLUT1+/- mice.** Anhedonic behavior measured by sucrose intake test following **(A)** 5 mg/kg ($F_{1.31}$=10.75 p=0.003) **(B)** 10 mg/kg ($F_{1.31}$=8.9 p<0.009) and **(C)** 15 mg/kg ($F_{1.31}$=1.56 p<0.04) in week 5. Ketamine was given once every 4 days. Data show mean ± SEM, sucrose intake (g/body weight), n=8-13 mice/group. ^^p<0.01, ^p<0.05 main effect of genotype (Two Way ANOVA).

Tail suspension test. The effect of ketamine (5, 10 or 15 mg/kg, i.p.) on immobility time was analyzed 60 minutes after the last drug injection. Interestingly, while ketamine revealed an anti-immobility action ($p<0.05$; $p<0.01$; $p<0.001$) in WT mice at the three doses tested, it was devoid of anti-immobility action in VGLUT1+/- mice **(Figure 24A)**.

Marble burying test. Marble burying test was performed in order to analyze the effect of ketamine in natural marble burying behavior. Ketamine (10 mg/kg, i.p.) reduced ($p<0.05$) the number of marbles buried 60 minutes after drug injection in WT but not in VGLUT1+/- mice **(Figure 24B)**.

Locomotor activity. WT and VGLUT1+/- mice were placed in an open field 60 minutes after ketamine (5, 10 or 15 mg/kg, i.p.) injection. Ketamine did not alter distance travelled between 60-90 minutes after the drug injection **(Figure 24C)**.

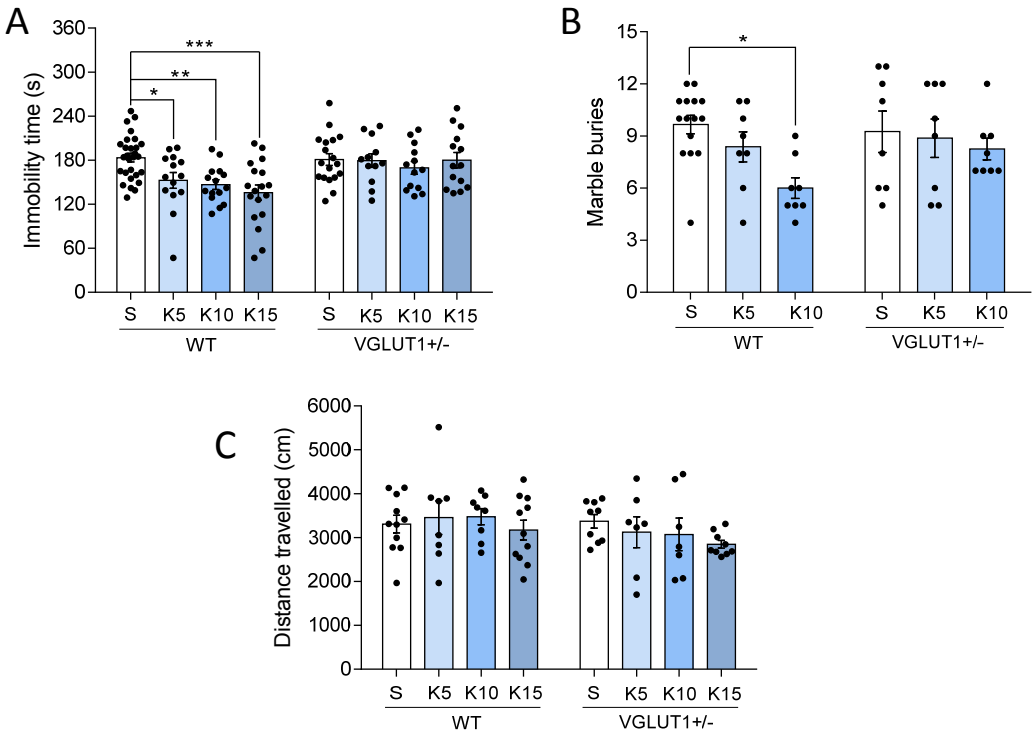

Figure 24. **Effect of ketamine in the helpless behavior of VGLUT1+/- mice. (A)** Helpless behaviour in the TST ($F_{3.124}$=2.912 p=0.03, significant interaction), **(B)** Marble burying behaviour ($F_{2.49}$=4.180 p= 0.02 significant interaction) and **(C)** locomotor activity. Ketamine was given at 5, 10 or 15 mg/kg, i.p. once every 4 days. Data show mean ± SEM, (A) immobility time (s) n=22-28 mice/group (B), number of marbles buried, n=11 mice/group and (C) distance travelled (cm), n=10 mice/group. ***p<0.0001, **p<0.01, *p<0.05 (Two Way ANOVA followed by Tukey *post-hoc* test).

2.2 Molecular effects of ketamine in the VGLUT1+/- depression model

We next studied the effect of the rapid antidepressant ketamine (10 mg/kg, i.p.) in PFC protein expression levels by western blot. Following four injections of ketamine (one every 4 days), PFC was dissected 90 minutes after the last drug injection. The effect of ketamine on the expression of glutamate targets, the synaptic plasticity marker pro-BDNF and the PI3K/Akt/mTOR intracellular signaling pathway were studied.

Effect of ketamine on the expression of VGLUT1, VGLUT2 and GluA1 in the VGLUT1+/- model.

As previously observed, VGLUT1 expression levels were reduced (p<0.001) in VGLUT1+/- mice. A main effect of treatment indicated that ketamine (10 mg/kg, i.p.) increased VGLUT1 (p<0.05) in both genotypes **(Figure 25A)**. Moreover, this rapid acting antidepressant did not affect VGLUT2 protein levels in the PFC **(Figure 25B)**. On the other hand, an increase (p<0.01) in the phosphorylated form of the AMPA receptor GluA1 subunit was observed in both genotypes following ketamine treatment compared to saline treated mice **(Figure 25C)**.

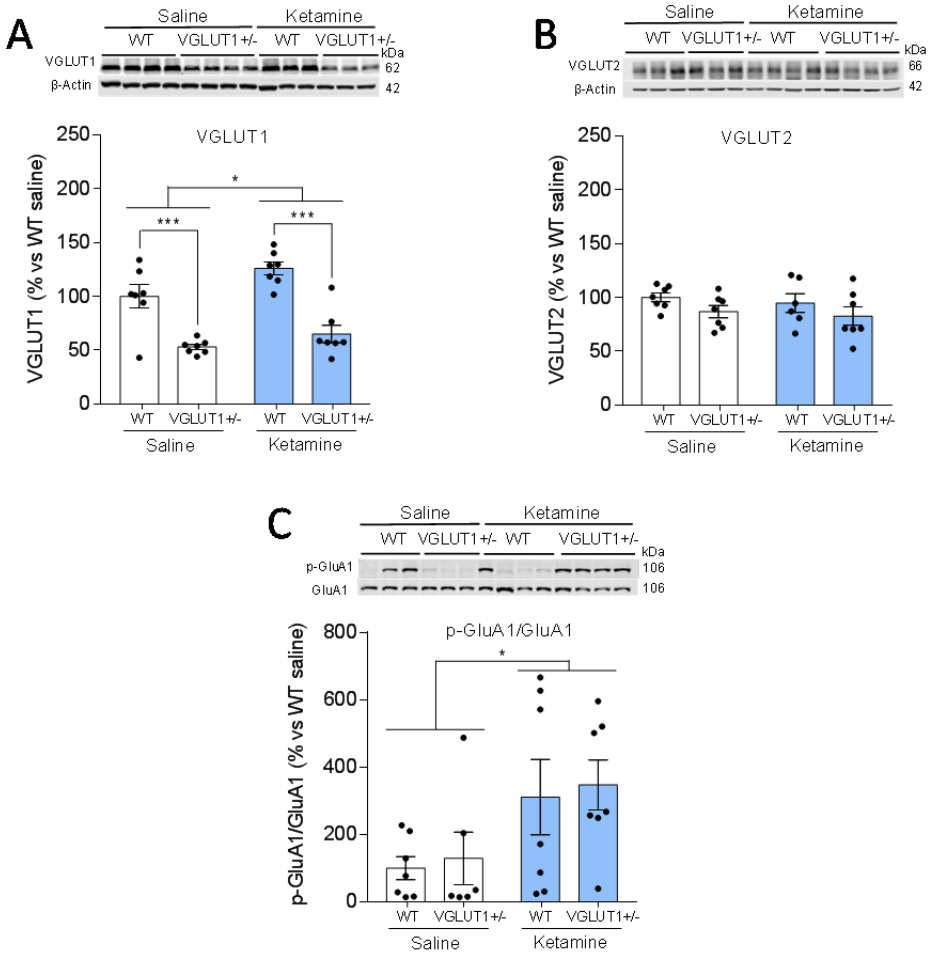

Figure 25. Ketamine regulation of glutamate targets. (A) PFC VGLUT1 ($F_{1.24}$=6.41 p=0.018, treatment $F_{1.24}$=51.49 p<0.0001, genotype), **(B)** VGLUT2 and **(C)** p-GluA1/GluA1 expression levels ($F_{1.24}$=7.18 p= 0.013) after treatment with ketamine (10 mg/kg, i.p.). Data show mean ± SEM, n=6-7 mice/group. ***p<0.001, **p<0.01 and *p<0.05, significant main effects (Two Way ANOVA).

Effect of ketamine on the expression of pro-BDNF in the VGLUT1+/- model.

Subsequently, it was checked whether the rapid acting antidepressant ketamine was able to upregulate pro-BDNF in the VGLUT1+/- model. Western blot analysis of pro-BDNF protein levels in the PFC, revealed a significant interaction between genotype and treatment ($p<0.05$). Posterior analysis revealed that ketamine (10 mg/kg, i.p.) was able to increase ($p<0.05$) pro-BDNF in the PFC of WT mice but not in VGLUT1+/- mice **(Figure 26)**.

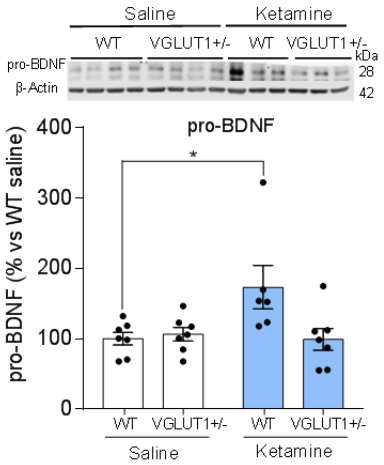

Figure 26. Effect of ketamine on PFC pro-BDNF expression in the VGLUT1+/- model. Pro-BDNF expression levels ($F_{1.23}$=5.43 p=0.028, signifciant interaction) after treatment with ketamine (10 mg/kg, i.p.). Data show mean ± SEM, n=6-7 mice/group.*p<0.05 (Two Way ANOVA, followed by Tukey *post-hoc* test).

Effect of ketamine on the expression of proteins involved in the PI3K/Akt/mTOR intracellular signaling pathway in the VGLUT1+/- model.

Ketamine (10 mg/kg, i.p.) treatment increased ($p<0.05$) phosphorylation of Akt in both genotypes compared to total levels **(Figure 27A)**. Yet, a significant interaction ($p<0.05$) between genotype and treatment ($p<0.01$) was found in p-ERK PFC expression levels. Specifically, this rapid acting antidepressant only increased ($p<0.05$) p-ERK in WT but not in VGLUT1+/- mice **(Figure 27B)**. Moreover, the phosphorylated fraction of mTOR protein was increased ($p<0.01$) after chronic treatment with ketamine in both genotypes **(Figure 27C)**. However, no changes on the phosphorylated ribosomal S6 occurred after treatment **(Figure 27D)**. Interestingly, a significant interaction ($p<0.01$) between ketamine treatment and genotype was found when measured phosphorylated eEF2. While ketamine showed a tendency to decreased phosphorylation of eEF2 protein in WT, it induced a striking increase in p-eEF2 ($p<0.05$) in the VGLUT1+/- mice **(Figure 27E)**.

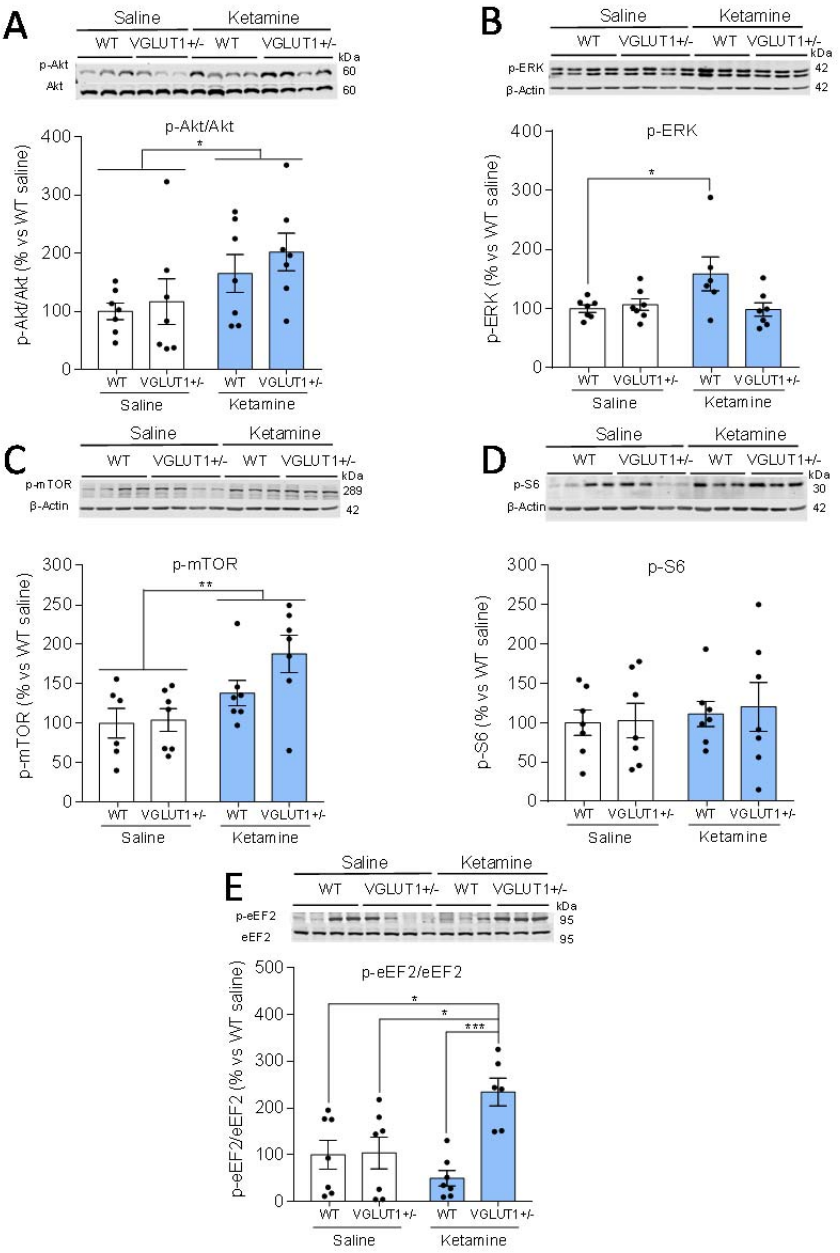

Figure 27. Ketamine regulation of the PI3K/Akt/mTOR intracellular signaling pathway in the VGLUT1+/- model. (A) p-Akt/Akt ($F_{1.24}$= 5.841 p=0.023), **(B)** p-ERK ($F_{1.23}$=4.88 p=0.037), **(C)** p-mTOR ($F_{1.23}$=10.75 p=0.003), **(D)** p-S6, **(E)** p-eEF2/eEF2 ($F_{1.23}$=9.94 p=0.004) expression levels after ketamine (10 mg/kg, i.p.) treatment. Data show mean ± SEM, n=6-7 mice/group for all proteins. **p<0.01, *p<0.05; main effect of treatment in A and C (Two Way ANOVA), significant interaction in B, E (Two Way ANOVA followed by Tukey *post-hoc* test).

2.3. Electrophysiological characterization of ketamine in the VGLUT1+/- model

In general the power spectral densities (PSDs) were characterized by a first high-intensity smooth delta (δ) and theta (θ) peak and then a descending slope to the higher frequencies gamma (γ) and HFO. As expected, brain activity was different between periods of stillness (<2 cm/s) and activity (>2cm/s). We ought to evaluate the existence

of differences in the PSD modulation resulting from ketamine (10 mg/kg, i.p.) administration in WT and VGLUT1+/- mice. To do that, we evaluated the corresponding 12 pre-injection minutes with the post-injection 12 minutes.

In the infralimbic cortex, WT and VGLUT1+/- mice showed different power spectrum profile. In both active and still periods, theta band intensity was higher for VGLUT1+/- compared to WT ($p<0.05$). Moreover, in the active period, frequency of theta band was lower in the heterozygous ($p<0.05$). Ketamine treatment decreased delta band intensity in both periods, and increased theta intensity ($p<0.01$) in stillness. Moreover, this drug decreased theta, gamma and HFO frequencies ($p<0.01$) in stillness. Interestingly, ketamine reduced HFO intensity specifically in the VGLUT1+/- mice during stillness ($p<0.05$) **(Figure 28 and 29)**.

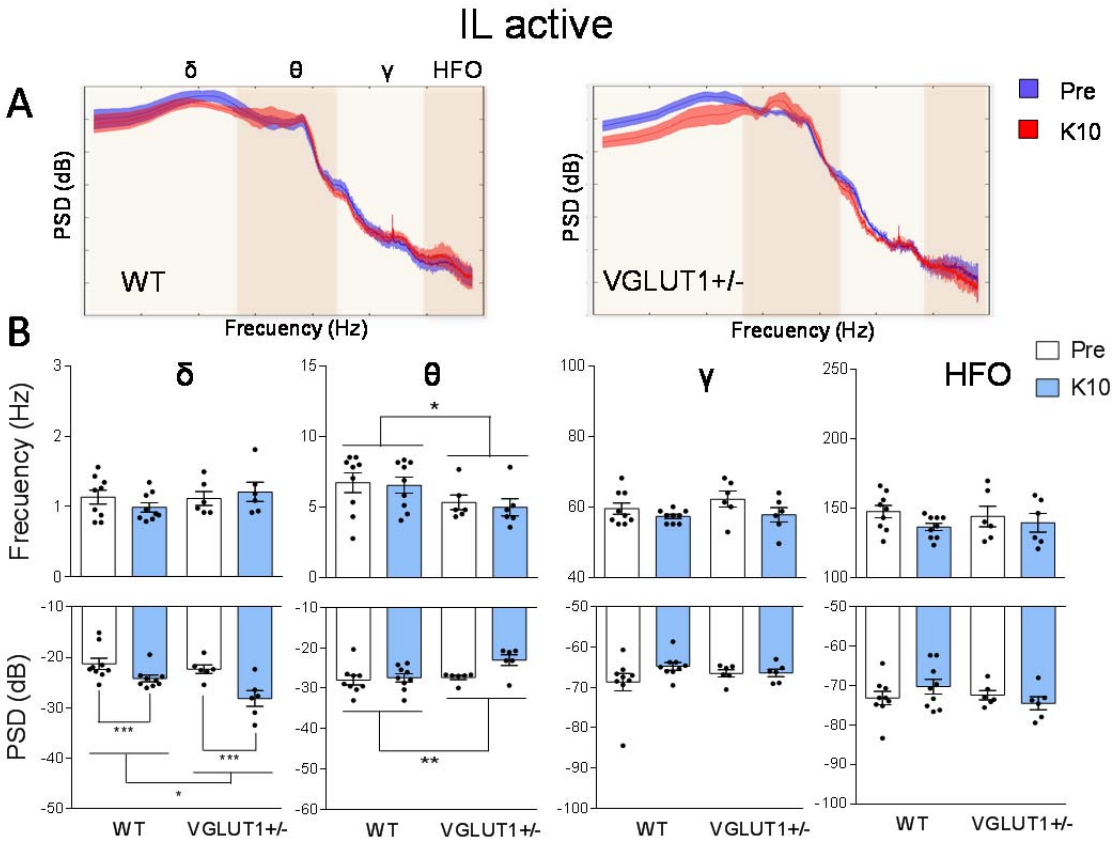

Figure 28. Effect of ketamine in the infralimbic cortex during active period. (A) Log-log representation of basal power histograms (PSD, power respect logHz, frequency) in IL of WT and VGLUT1+/- mice. **(B)** Histograms showing frequencies (upper) and intensities (lower) for delta, theta, gamma and HFO bands. In delta, intensity ($F_{1.13}$= 4.48 p=0.05, genotype and $F_{1.13}$= 21.81 p=0.001, treatment). In theta, ($F_{1.13}$=5.26 p=0.03 and $F_{1.13}$=9.48 p=0.008, genotype, frequency and intensity, respectively). Data show the mean ± SEM, frequencies in Hz and PSD in dB. ***p<0.001, **p<0.01, *p<0.05 main effects (Two Way ANOVA).

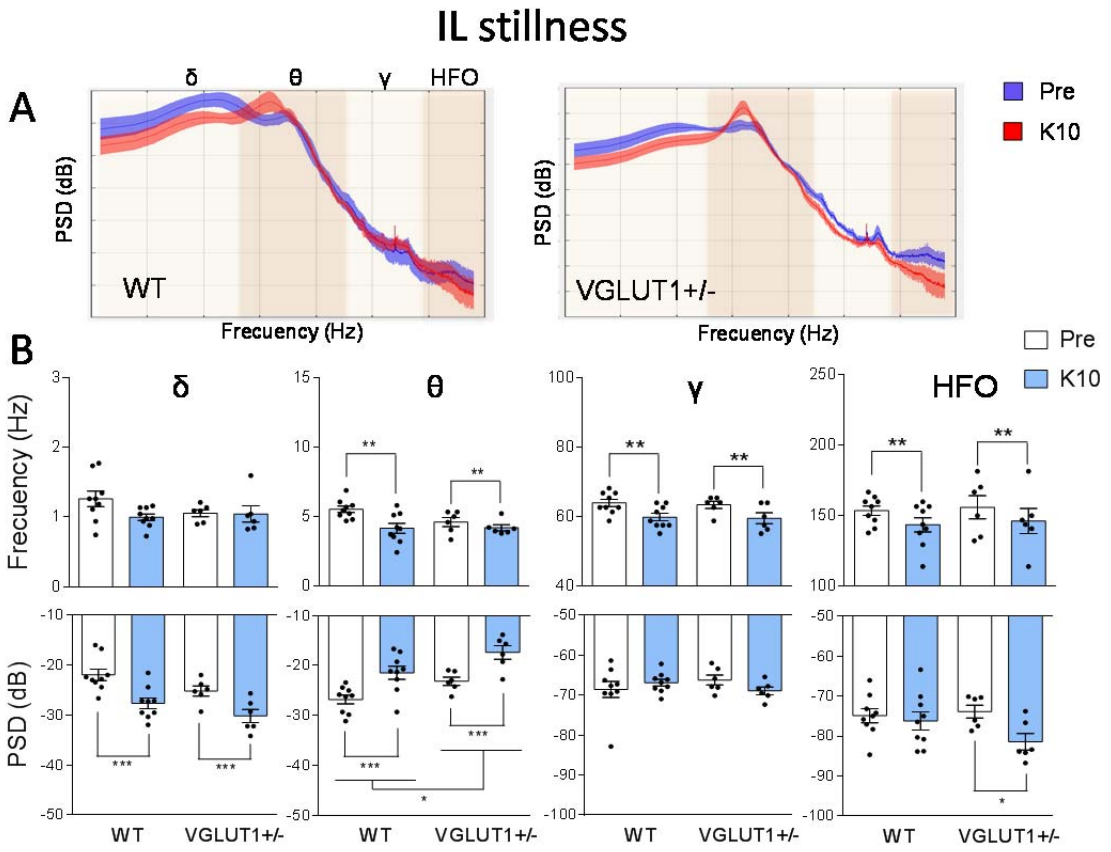

Figure 29: Effect of ketamine in the infralimbic cortex during stillness. (A) Log-log representation of basal power histograms (PSD, power respect logHz, frequency) in IL of WT and VGLUT1+/- mice. **(B)** Histograms showing frequencies (upper) and intensities (lower) for delta, theta, gamma and HFO bands. In delta ($F_{1.13}$= 67.29 p=0.0001, main effect). In theta ($F_{1.13}$=10.42 p=0.006) for frequency and ($F_{1.13}$= 41.26 p<0.0001, genotype; $F_{1.13}$= 7.73 p=0.015, treatment) for intensity. In gamma ($F_{1.13}$=9.70 p=0.008) for frequency. In HFO ($F_{1.13}$=7.52 p=0.01) for frecuency and ($F_{1.13}$=4.28 p=0.05, interaction) for intensity. Data show the mean ± SEM, frequencies in Hz and PSD in dB. ***p<0.001, **p<0.01, *p<0.05 main effects (Two Way ANOVA followed by Sidak *post-hoc* test).

In the frontal asociation area, ketamine treatment decreased delta band intensity and increased both theta and gamma bands intensities (p<0.01) during active and still periods. Moreover, this drug decreased theta and HFO frequencies in stillness in both genotypes (p<0.01). Interestingly, ketamine (10mg/kg, i.p.) reduced HFO frequency (p<0.01) period and show a tendency to increase HFO intensity specifically in the WT mice but not in the VGLUT1+/- in the active period **(Figure 30 and 31)**.

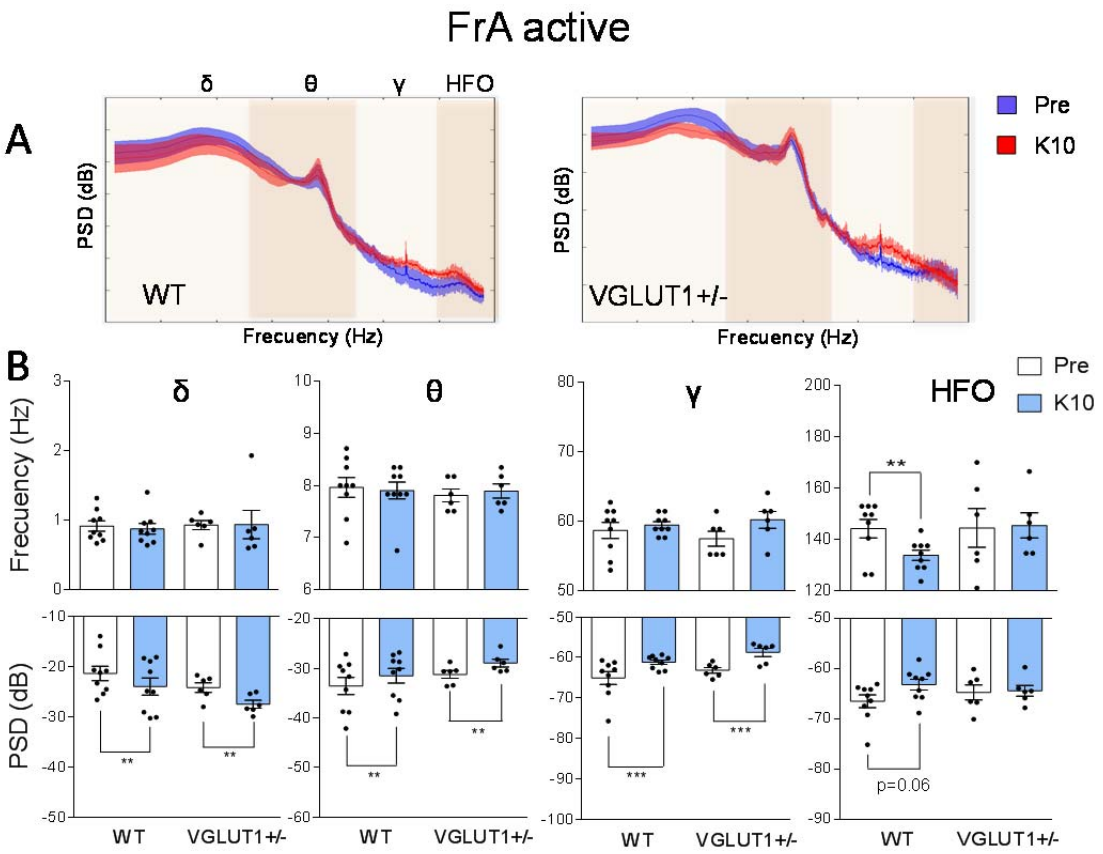

Figure 30: Effect of ketamine in the frontal asociation cortex during active. (A) Log-log representation histograms (PSD, power respect logHz, frequency) in FrA of WT and VGLUT1+/- mice. **(B)** Histograms showing frequencies (upper) and intensities (lower). In delta ($F_{1.13}$=13.01 p=0.003), theta ($F_{1.13}$=14.89 p=0.002) and gamma ($F_{1.13}$=18.62 p=0.001) effect of treatment. In HFO ($F_{1.13}$=5.74 p=0.008, interaction) in frequency. Data show the mean ± SEM, frequencies in Hz and PSD in dB. ***p<0.001, **p<0.01, main effects (Two Way ANOVA followed by Sidak *post-hoc* test).

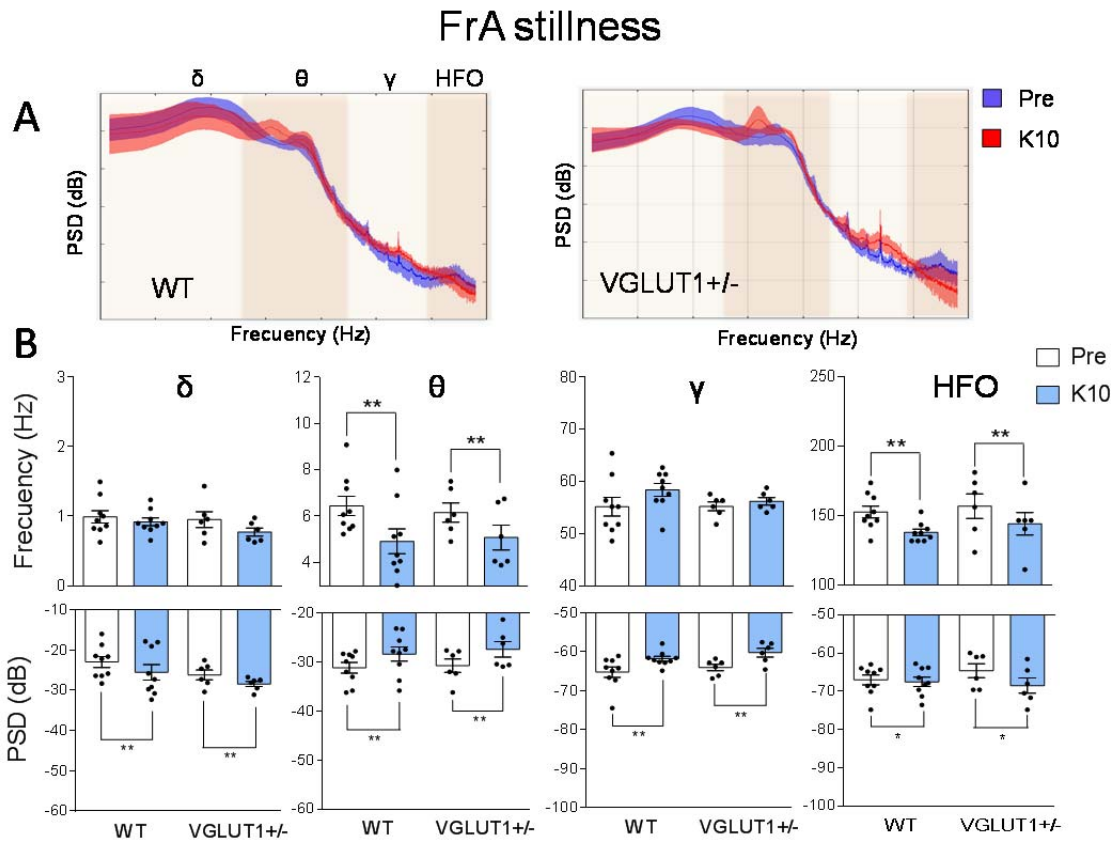

Figure 31: Effect of ketamine in the frontal asociation cortex during stillness. (A) Log-log representation histograms (PSD, power respect logHz, frequency) in FrA of WT and VGLUT1+/- mice. **(B)** Histograms showing frequencies (upper) and intensities (lower). In delta ($F_{1.13}$=10.3 p=0.008, main effect), theta ($F_{1.13}$=7.78 p=0.01, for frequency), ($F_{1.13}$= 6.39 p<0.02, for intensity) and gamma ($F_{1.13}$=16.96 p=0.001, main effect of treatment). In HFO ($F_{1.13}$=26.16 p=0.0001, for frequency) and ($F_{1.13}$=6.75 p=0.02, intesity). Data show the mean ± SEM, frequencies in Hz and PSD in dB. ***p<0.001, **p<0.01, *p<0.05, main effects (Two Way ANOVA).

3. Rescue of the rapid antidepressant action of ketamine in VGLUT1+/- model

3.1 Rapid antidepressant action of ketamine in the VGLUT1+/- model pretreated with reboxetine

Since VGLUT1 levels were restored after a chronic treatment with reboxetine (15 mg/kg, i.p.), we next studied the effect of ketamine (10 mg/kg, i.p.) in the depressive-like behavior of VGLUT1+/- mice pretreated with this classic antidepressant.

Reboxetine pretreatment rescued anhedonic behavior of VGLUT1+/- mice.

After two basal intake measurements, VGLUT1+/- mice showed lower (p<0.05) sucrose intake compared to WT as expected. Subsequently, four week treatment with

reboxetine (15 mg/kg, i.p.) increased significantly (p<0.05) sucrose intake in both genotypes rescuing anhedonic behavior of VGLUT1+/- **(Figure 32A)**.

Reboxetine pretreatment rescued anti-helpless activity of acute ketamine in the VGLUT1+/- model.

After three days from the last administration with reboxetine, the acute effect of saline or ketamine (10 mg/kg, i.p.) was tested in the TST, performed 1h after drug injection. A significant effect of treatment was found (p<0.05). Both in WT (p<0.01) and VGLUT1+/- (p<0.05) mice pretreated with reboxetine and with an acute injection of ketamine presented a significant reduction in immobility time. However, washed saline treated mice with an acute ketamine injection reduced (p<0.05) the immobility time in WT but not in VGLUT1+/- mice as previously shown **(Figure 32B)**.

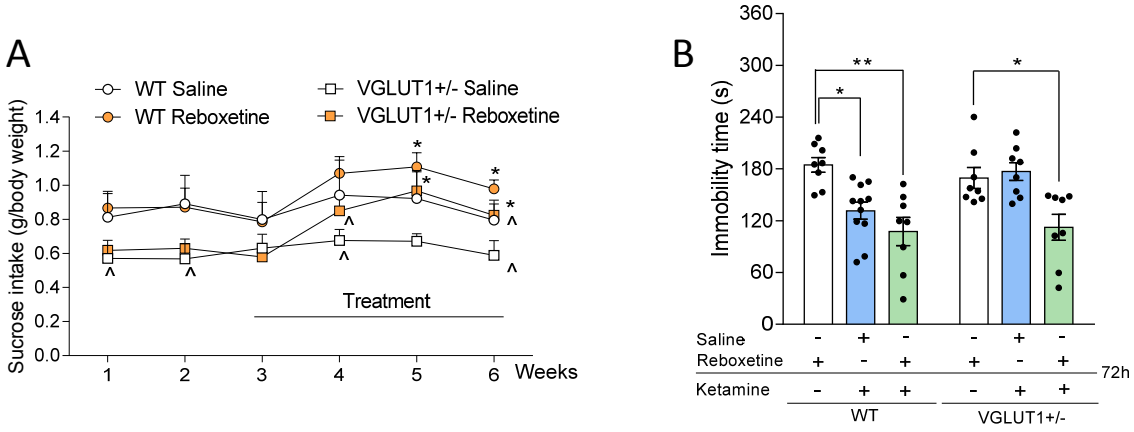

Figure 32. Rapid antidepressant action of ketamine in the VGLUT1+/- model pretreated with reboxetine. (A) Sucrose intake following chronic reboxetine treatment ($F_{1.44}$=5.12 p<0.05, genotype and $F_{1.44}$= 6.84 p<0.05, treatment in week 6). **(B)** Helpless behavior following acute injection with ketamine 10 mg/kg, i.p. ($F_{2.45}$=3.303 p=0.02, significant interaction). Data show mean ± SEM, (A) sucrose intake (g/body weight) or (B) immobility time (s), n=8-11 mice/group. *p<0.05; main effect of treatment, ^p<0.05; main effect of genotype in A; **p<0.01, *p<0.05 in B (Two Way ANOVA followed by Tukey *post-hoc* test).

Chronic reboxetine pretreatment rescues VGLUT1 and the effect of ketamine in the p-eEF2/eEF2 ratio in the VGLUT1+/- model.

Chronic reboxetine pretreatment for four weeks and following 72h of washout period induced a significant increase of VGLUT1 protein levels in the PFC in both genotypes (p<0.05) **(Figure 33A)**. Moreover, after the chronic treatment, acute treatment with ketamine (10mg/kg, i.p.) dephosphorylated p-eEF2 (p<0.05) in WT and VGLUT1+/- mice model 90 minutes after drug injection **(Figure 33B)**.

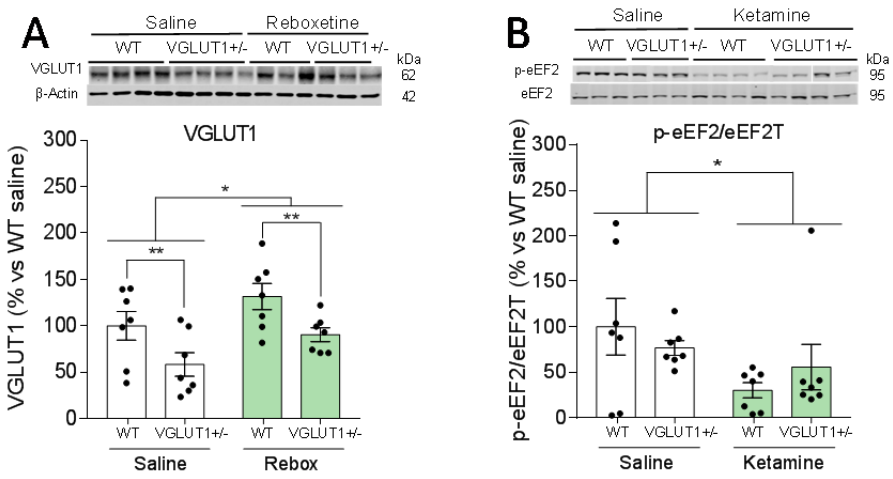

Figure 33. Chronic reboxetine pretreatment rescues VGLUT1 and the effect of ketamine in the p-eEF2/eEF2 ratio in the VGLUT1+/- model. (A) PFC VGLUT1 expression levels following chronic reboxetine pretreatment ($F_{1.24}$=6.217 p=0.019) **(B)** p-eEF2/eEF2 expression levels following acute injection with ketamine (10 mg/kg, i.p.) ($F_{1.24}$=4.758 p=0.0392) following chronic reboxetine pretreatment. Reboxetine was administered for four weeks at 15 mg/kg and was followed by a 72h whashout period. Data show mean ± SEM, n=7 mice/group. **p<0.01, *p<0.05; main effects (Two Way ANOVA).

3.2 Rapid antidepressant action of ketamine in the VGLUT1+/- model with PFC VGLUT1 induced expression

Subsequently, we studied how VGLUT1 levels in the PFC could influence the antidepressant action of ketamine in the VGLUT1+/- model. Using adeno-associated virus (AAV) technology, AAV-pSyn-VGLUT1mCherryminisog (VGLUT1^{mCherryminisog}) and AAV-pSyn-sVGLUT1mCherryminisog (sVGLUT1^{mCherryminisog}) were injected in the PFC of mice. In addition, AAV-pSyn-YFP (YFP) was used as an internal control.

Imaging of VGLUT1^{mCherryminisog}, sVGLUT1^{mCherryminisog} and YFP in the PFC of the VGLUT1+/- model.

In vivo gene transfer efficacy was determined by imaging analysis using an epifluorescence microscope with a stitching method. Fluorescence was visible in serial sliced sections of the PFC of mouse brain injected with VGLUT1^{mCherryminisog}, sVGLUT1^{mCherryminisog} and YFP under synapsin promoter. Immunofluorescence was performed 4 weeks after injection (**Figure 34A, B and C)**. At higher magnification ectopic VGLUT1^{mCherryminisog} and sVGLUT1^{mCherryminisog} expression was visualized in neuronal soma and the neuropil (**Figure 34A´, B´ and C´)**. No changes in the amount of total immunofluorescence were detected between the two genotypes indicating that AAV efficiency is similar in both **(Figure 34D)**. Western blot analysis confirmed the

presence of endogenous PFC VGLUT1 (60 kDa) and VGLUT1^{mCherryminisog} induced by the AAV (~100 kDa) in both WT and VGLUT1+/- mice injected with AAV-pSyn-VGLUT1mCherryminisog **(Figure 34E)**.

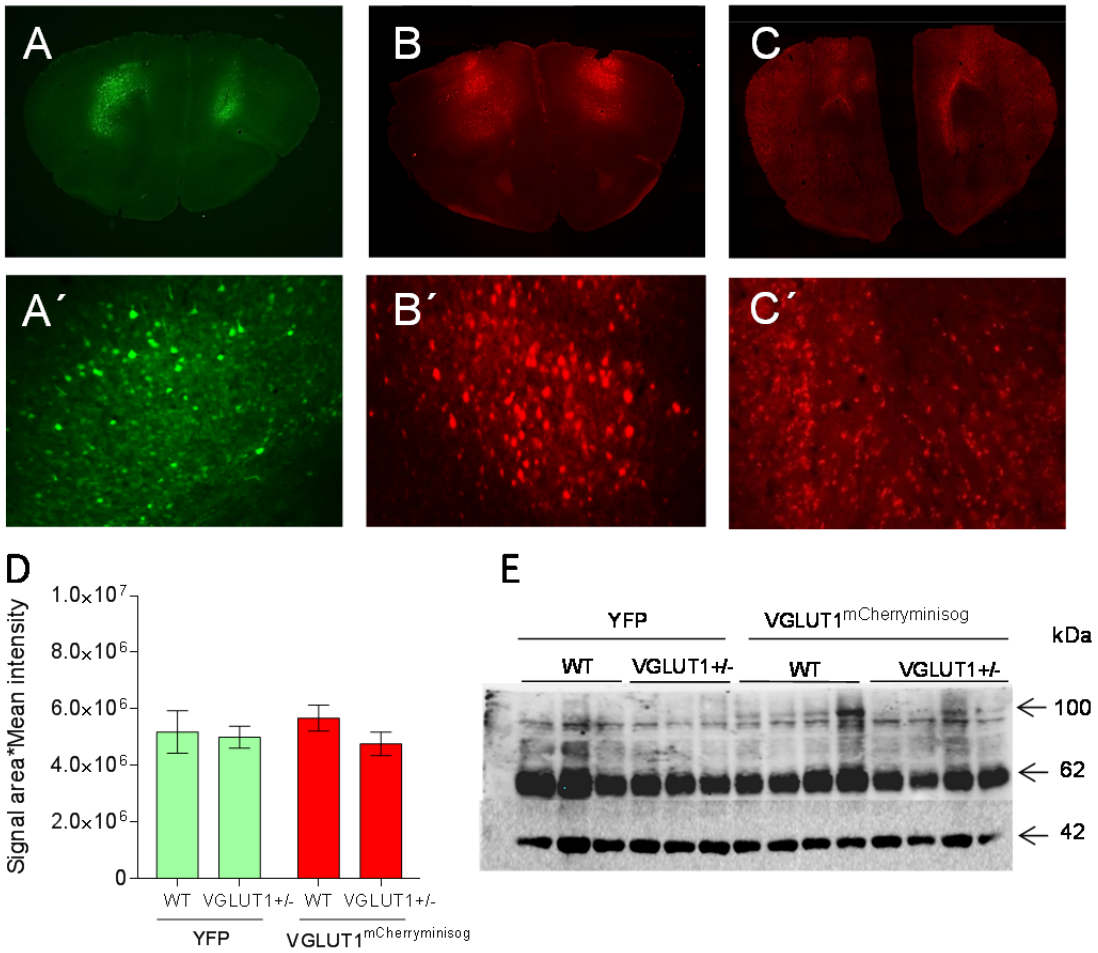

Figure 34. Adeno-associated virus VGLUT1^{mCherryminisog} and glutamate transport deficient mutant sVGLUT1^{mCherryminisog} expression in the PFC. Representative expression of **(A)** YFP **(B)** VGLUT1^{mCherryminisog} and **(C)** sVGLUT1^{mCherryminisog} at low 4X and **(A'-C')** high 20X magnification. In A, B and C scale bar means 1 mm and in A', B' and C´ means 50 µm. **(D)** Quantification of AAV efficiency (score signal area * intensity) in the PFC of WT and VGLUT1+/- mice. **(E)** Representative immunoblots of endogenous PFC VGLUT1 (60 kDa), VGLUT1^{mCherryminisog} induced by the AAV (≈100 kDa) and β-Actin (42 kDa) in both WT and VGLUT1+/- mice.

Effect of PFC VGLUT1^{mCherryminisog} *and sVGLUT1*^{mCherryminisog} *induced expression in anhedonic-like behavior of the VGLUT1+/- model.*

Before to the AAV injection, the intake baseline for the sucrose solution was established, corresponding to the average of three consecutive measurements. A significant effect of genotype was observed ($p<0.05$) along the weeks between WT and VGLUT1+/- mice. Each genotype was divided in three groups matched for sucrose consumption and body weight. Importantly, VGLUT1^{mCherryminisog} injection increased sucrose intake on second ($p<0.05$), third and fourth ($p<0.01$) week after surgery in both genotypes, rescuing normal levels in VGLUT1+/- mice **(Figure 35A)**. On the other hand, sVGLUT1^{mCherryminisog} did not affect to the sucrose intake **(Figure 35B)**.

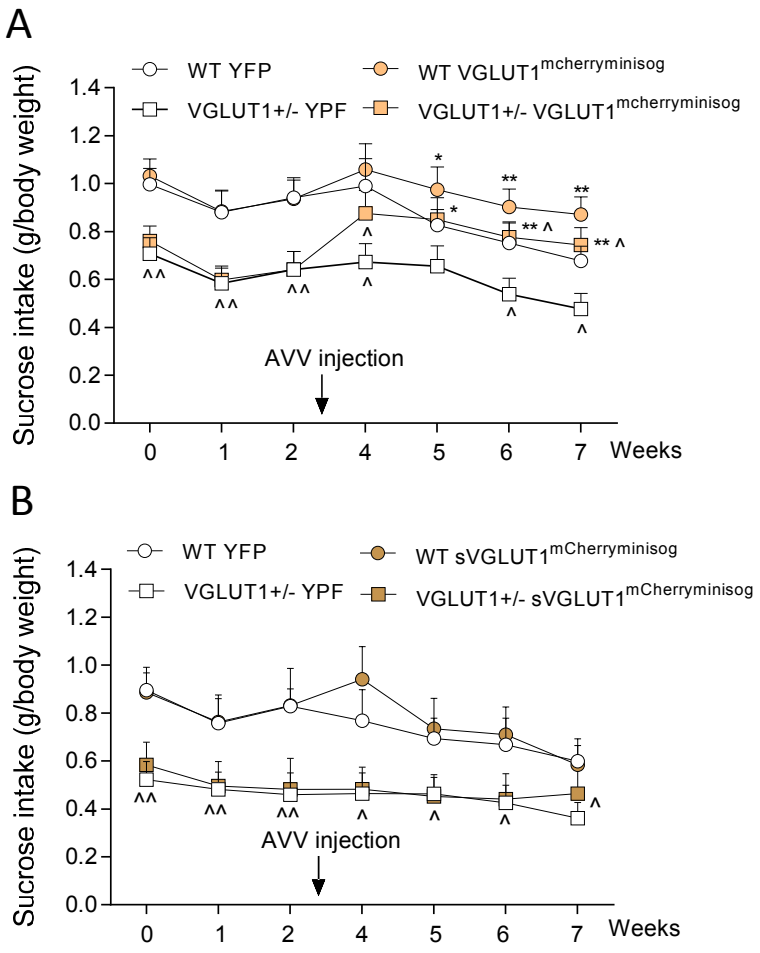

Figure 35. **Effect of PFC VGLUT1**^{mCherryminisog} **and sVGLUT1**^{mCherryminisog} **induced expression in anhedonic-like behavior of VGLUT1+/- mice.** Anhedonic behavior following PFC injection of (**A)** VGLUT1^{mCherryminisog} ($F_{1.44}$=11.6 p=0.001, treatment and $F_{1.44}$=5.52 p=0.02, genotype effect for week 7) and **(B)** sVGLUT1^{mCherryminisog} ($F_{1.29}$=6.10 p=0.04, genotype effect for week 7). Data show mean ± SEM, sucrose intake (g/body weight), n=18-21 mice/group for A, n=8-9 mice/group for B. **p<0.01, *p<0.05; main effect of treatment, ^^p<0.01, ^p<0.05; main effect of genotype (Two Way ANOVA).

Anti-helpless action of ketamine is rescued in the VGLUT1+/- model with VGLUT1 induced expression.

TST was performed 1h after a single dose of saline or ketamine (10 mg/kg, i.p.) in WT and VGLUT1+/- mice injected with VGLUT1^{mCherryminisog}, sVGLUT1^{mCherryminisog} or YFP. Yet, acute ketamine administration reduced ($p<0.05$) immobility time in all subgroups of WT mice. In addition, ketamine reduced immobility time ($p<0.05$) in VGLUT1+/- mice with PFC VGLUT1^{mCherryminisog} induced expression **(Figure 36A)** but it showed no effect in VGLUT1+/- mice with induced expression of sVGLUT1^{mCherryminisog} **(Figure 36B)**. This result indicates that VGLUT1 induced expression in the PFC rescues the anti-helpless effect of ketamine in the VGLUT1+/- model.

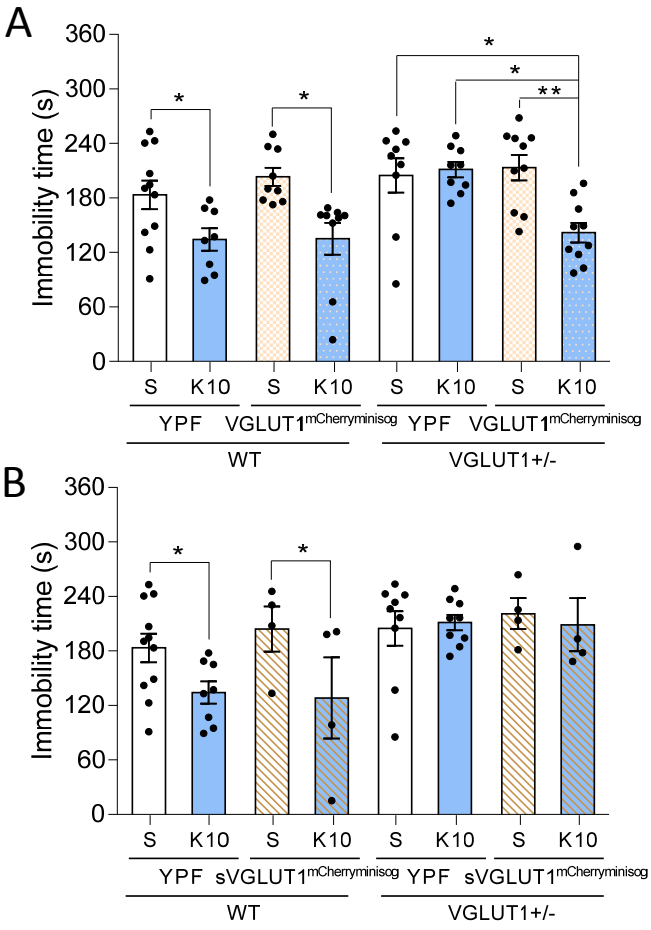

Figure 36. **VGLUT1^{mCherryminisog} induced expression rescues the anti-helpless action of ketamine in the VGLUT1+/- model**. **(A)** Helpless behavior in the TST of mice with PFC VGLUT1^{mCherryminisog} induced expression ($F_{3.67}=3.258$ $p=0.026$, significant interaction) and **(B)** mice with PFC sVGLUT1^{mCherryminisog} induced expression ($F_{1.23}=7.49$ $p=0.011$, effect of treatment). Ketamine was given acutely at 10 mg/kg, i.p. Data show mean ± SEM of immobility time (s) of n=9-11 mice/group for A, n=4-9 mice/group for B. **p<0.01, *p<0.05, significant interaction (Two Way ANOVA followed by Tukey *post-hoc* test).

4. The melatonin 2 receptor knock-out model of depression: behavioral features, circadian temperature rhythm and antidepressant treatment

4.1 Depressive-like behavior and altered circadian temperature of the MT_2-/- mice.

Depressive-like behavior. We first studied the effect of genotype on depressive-like behavior and on 24h circadian CBT rhythm. Firstly, three basal measurements were taken together in order to analyze anhedonic behavior in MT_1-/- and MT_2-/- mice. Despite MT_1-/- mice not showing significant decrease in sucrose intake, MT_2-/- mice showed a significant decrease ($p<0.01$) compared to WT littermates **(Figure 37A)**. In the novel object-induced CPP test, while WT and MT_1-/- mice became clearly conditioned towards the compartment with daily placement of new objects, MT_2-/- mice showed no preference for any of the two compartments ($p<0.05$) **(Figure 37B)**. Further, MT_2-/- mice did not respond to the hypothermic action of the 5-HT_{1A} agonist 8-OH-DPAT as did WT ($p<0.01$) and MT_1-/- ($p<0.05$) littermates **(Figure 37C)**.

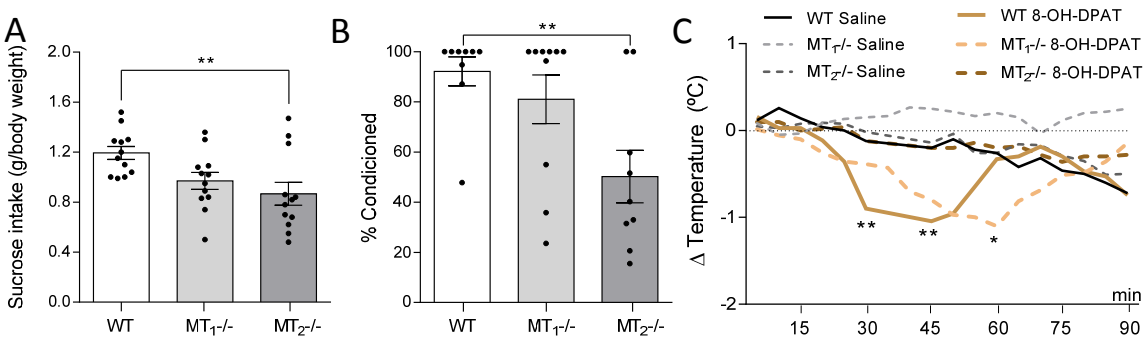

Figure 37. MT_2-/- mice show depressive-like behavior. **(A)** Anhedonic-like behavior measured with the sucrose intake test (g/body weight, 6 pm to 9 am) ($F_{2.33}$=5.29 p=0.0101), **(B)** Novel object-induced CPP measured by % time spent in the conditioned compartment ($F_{2.25}$=5.72 p= 0.009) and **(C)** 5-HT_{1A} agonist induced hypothermic effect using single dose of 8-OH-DPAT (0.25 mg/kg s.c.) or saline on core body temperature. Data show mean ± SEM n=11-12/group for A, B and C. **p<0.01, *p<0.05; main effect of genotype (Two Way ANOVA).

Altered circadian core body temperature. On the other hand, average of circadian CBT was recorded every 5 minutes along four weeks and differences among genotypes were found. MT_2-/- mice showed a significant decrease ($p<0.01$) in the AUC defined by the 24h light-dark (sleep-wake) period of body temperatures compared to WT and MT_1-/- littermates **(Figure 38A and B)**. Further analysis of the circadian curve in 2 h-fractions revealed that MT_2-/- mice showed lower CBT ($p<0.01$) compared to WT with the exception of the last 4h of the dark cycle (from 4 am till 8 am). Moreover, MT_1-/-

mice showed lower (p<0.05) CBT compared to WT in some fractions**.** Further, a significant *Pearson's* correlation between sucrose intake and 24h CBT was found (0.435, p<0.05) (Data not shown)**.**

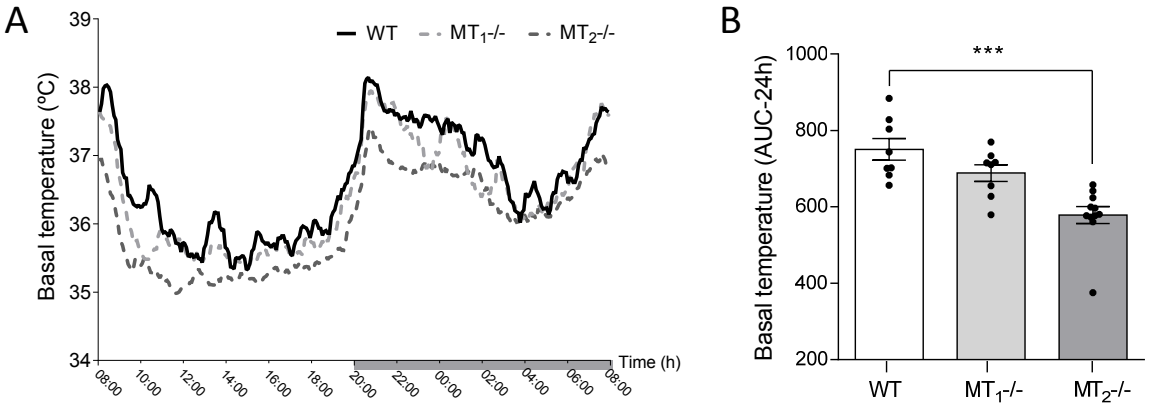

Figure 38. MT_2-/- mice show altered circadian CBT. **(A)** Representative image of 24h circadian CBT (°C) recordings (every 5 min). **(B)** Average of three weekly AUC values of circadian CBT ($F_{2.24}$=13.70 p=0.0001). Data show mean ± SEM of n=11-12 mice/group for A and B. ***p<0.01 *vs.* WT mice (One-Way ANOVA followed by Tukey *post-hoc* test).

Reward exposure. In order to study mice sensitivity to reward exposure, dark period CBT during sucrose intake test was measured in WT and MT_2-/- mice and compared to CBT in a standard night. Mean temperature differences between the night in which sucrose intake test was performed and a standard night were higher (p<0.05) for WT than for MT_2-/- mice **(Figure 39A and B)**.

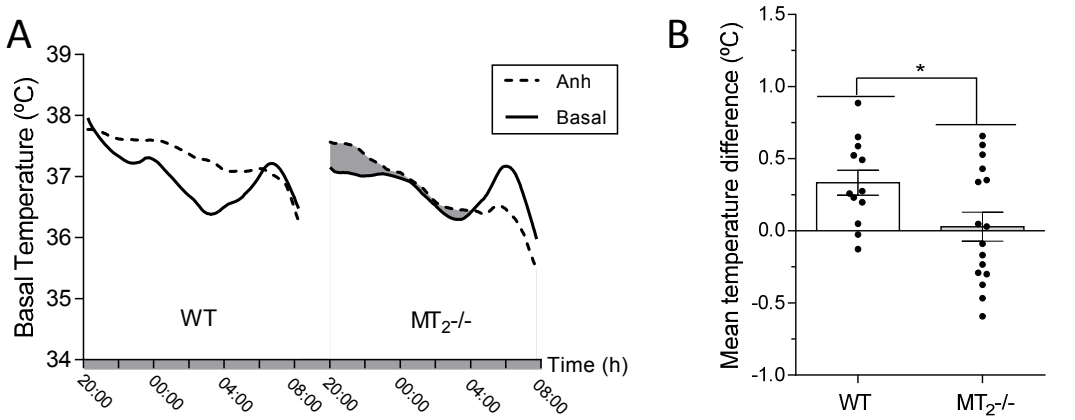

Figure 39. Reward exposure alters differently dark circadian CBT in WT and MT_2-/- mice. (A) Representative images of circadian CBT recordings in the dark period in standard conditions (grey line) and during a rewarding night (dark line) for WT and MT_2-/- mice. **(B)** Mean temperature differences between rewarding and standard night for WT and MT_2-/- mice (p=0.037). Data show mean ± SEM; n=12-16 mice/group for A and B. *p<0.05 MT2-/- *vs* WT (Student-t test). A local regression method (robust LOESS) was used to smooth the data represented in A, in order to remove noise-like features and emphasize significant trends.

Social interaction. We also studied the different vulnerability of WT, MT_1-/- and MT_2-/- mice to chronic stress using the CSDS model. CSDS induced a decrease in social interaction in all mice ($p<0.001$). In addition, MT_2-/- defeated mice had an increased social avoidance ($p<0.05$) compared to defeated WT **(Figure 40)**.

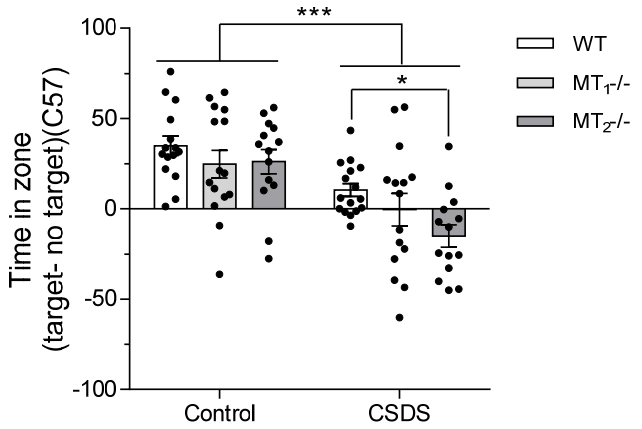

Figure 40. MT_2-/- mice show more vulnerability to chronic social defeat stress in the social interaction test. Difference of time spent in the interaction zone in the social interaction test (target-without target) by the effect of stress ($F_{1.83}$=31.34 $p<0.0001$) or genotype ($F_{2.83}$=3.42 $p= 0.037$). Data show mean ± SEM of n=14-16 mice/group. ****p*<0.001; main effect of stress, **p*<0.05; MT_2-/- CSDS vs WT CSDS (Two Way ANOVA followed by Tukey *post-hoc* test).

4.2 Effect of the antidepressant reboxetine in depressive-like behavior and altered circadian CBT of MT_2-/- mice

Depressive-like behavior. The effect of chronic reboxetine (15 mg/kg, i.p.) on depressive-like behavior and on altered circadian CBT of MT_2-/- mice was studied**.** On the third week of treatment, the classic antidepressant treatment increased sucrose intake ($p<0.05$) rescuing normal levels **(Figure 41A)** and revealed an anti-immobility action in the TST ($p<0.01$) in both genotypes 60 minutes after drug injection **(Figure 41B)**. On the fourth week, it failed to rescue MT_2-/- mice lack of preference in the novel object induced CPP **(Figure 41C)**. Finally, chronic reboxetine treatment rescued 5-HT_{1A} agonist induced hypothermia in MT_2-/- mice at 30, 45 and 60 min after 8-OH-DPAT injection ($p<0.01$) **(Figure 41D)**.

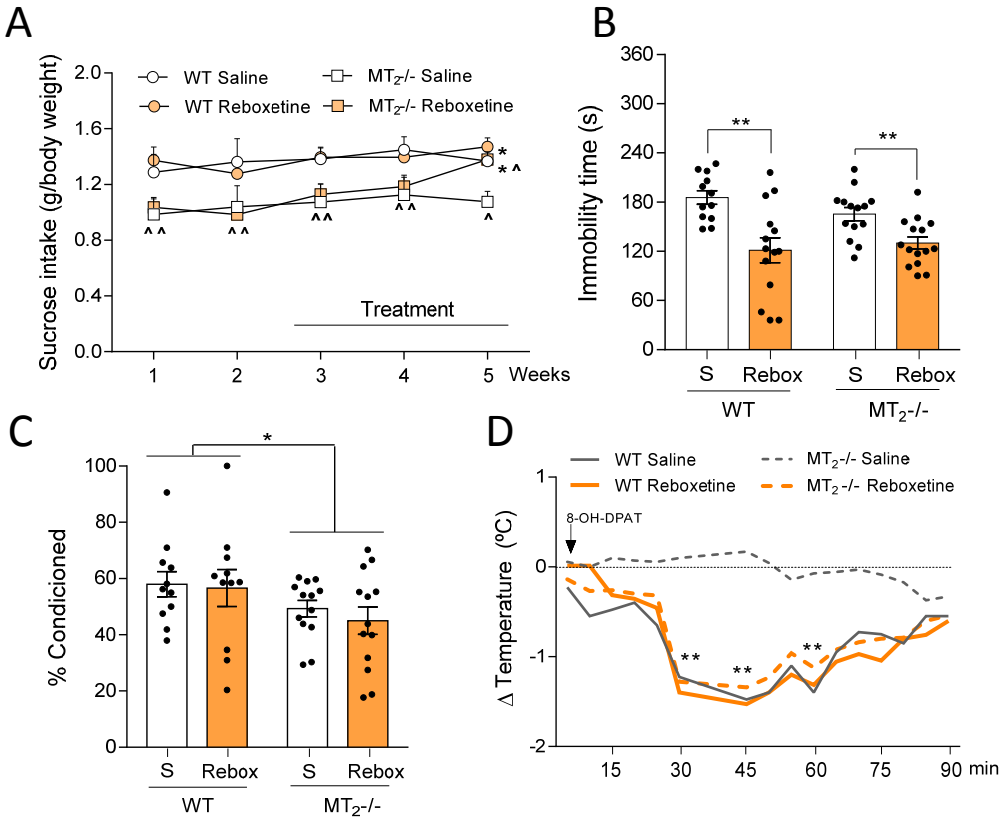

Figure 41. Depressive like behavior and altered circadian CBT of MT_2-/- mice is reverted by antidepressant treatment. (A) Anhedonic behavior was measured with the sucrose intake test ($F_{1.34}$= 4.40 p=0.43) **(B)** Immobility time (s) on the TST one hour after drug injection ($F_{1.51}$=23.13 p=0.0001), **(C)** Novel object-induced CPP measured by % time spent in the conditioned compartment ($F_{1.44}$= 4.53 p=0.038) and **(D)** 5-HT_{1A} agonist induced hypothermic effect using single dose of 8-OH-DPAT or saline after injection ($F_{3.27}$=9.24 p=0.001). Data show mean ± SEM; n=12-15 mice/group for A, B and C, n=10 mice/group for D. **p<0.01; main effect of treatment, *p<0.05; main effect of genotype (Two Way ANOVA).

Altered circadian core body temperature. On the other hand, 24h circadian core body temperature rhythm curves every 15 minutes were obtained on the third week of chronic reboxetine (15 mg/kg, i.p.) treatment in WT and MT_2-/- mice. As shown in **(Figure 42A)**, MT_2-/- mice presented a significant decrease in 24h AUC of CBT (p<0.05) as previously shown and importantly, chronic reboxetine treatment increased 24h AUC of CBT in both genotypes (p<0.05) **(Figure 42B)**.

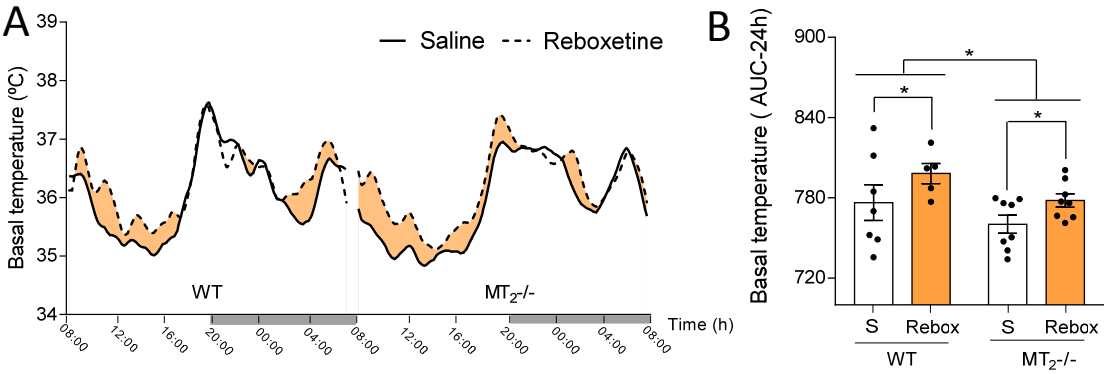

Figure 42. Altered circadian CBT of MT_2-/- is reverted by antidepressant treatment. (A) Representative image of circadian CBT recordings (every 15 minutes). **(B)** AUC values for WT and MT_2-/- mice ($F_{1.24}$=4.27 p=0.049) treated with saline or reboxetine for three weeks ($F_{1.24}$=5.06 p=0.034). Data show mean ± SEM; n=5-8 mice/group for A and B. *p<0.05; main effect of treatment and genotype (Two Way ANOVA). A local regression method (robust LOESS) was used.

Reward exposure. Chronic reboxetine treatment increased significantly (p<0.05) mean temperature diference in WT and MT_2-/- mice in the sucrose intake during dark period **(Figure 43A, B and C)**.

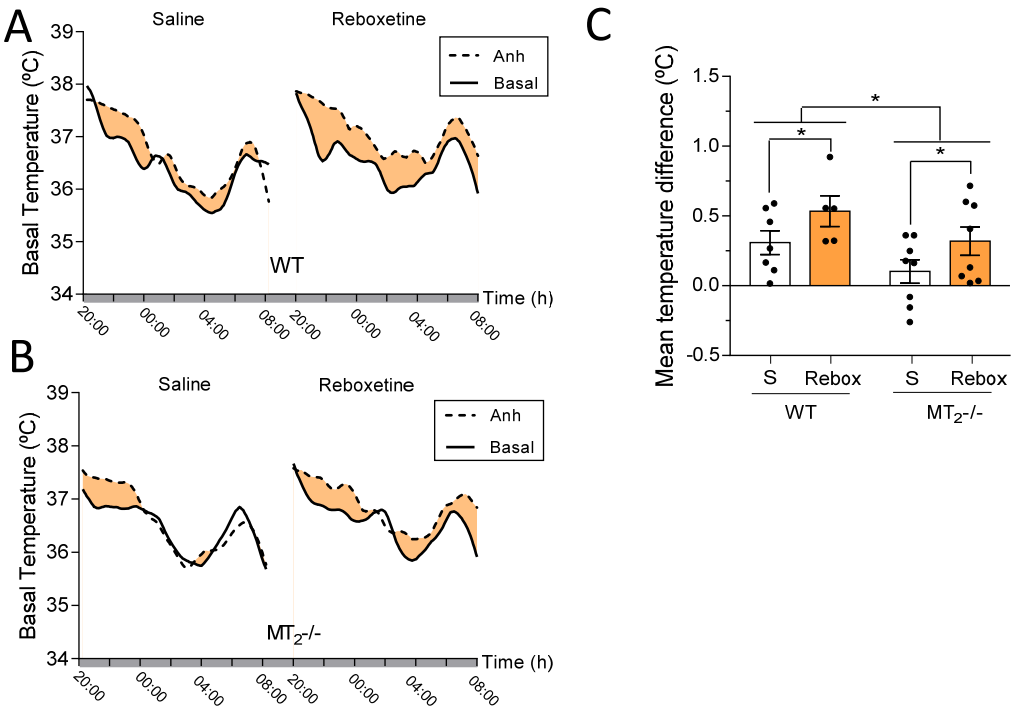

Figure 43. Dark circadian CBT is increased after chronic antidepressant treatment in WT and MT_2-/- mice. (A, B) Representative images of circadian CBT recordings in the dark period on the third week of treatment during standard conditions (grey line) and during reward exposure (dark line). **(C)** Mean temperature differences between rewarding and standard night ($F_{1.24}$=5.06 p=0.034). Data show mean ± SEM, n=5-8 mice/group for C. *p<0.05 main effect of treatment and genotype (Two Way ANOVA). A local regression method (robust LOESS) was used.

4.3. The rapid acting antidepressant ketamine reverses depressive-like behavior of MT_2-/- mice

Depressive-like behavior. The rapid antidepressant action of ketamine (15 mg/kg, i.p.) was also studied in the MT_2-/- model. After three basal measurements, MT_2-/- mice showed anhedonic-like behavior ($p<0.01$) as previously presented. Importantly, ketamine treated mice increased sucrose intake ($p<0.05$) after three injections in WT and MT_2-/- mice **(Figure 44A)**. In addition, ketamine showed an anti-immobility effect ($p<0.01$) in the TST 60 minutes after the last drug injection in both genotypes **(Figure 44B)**.

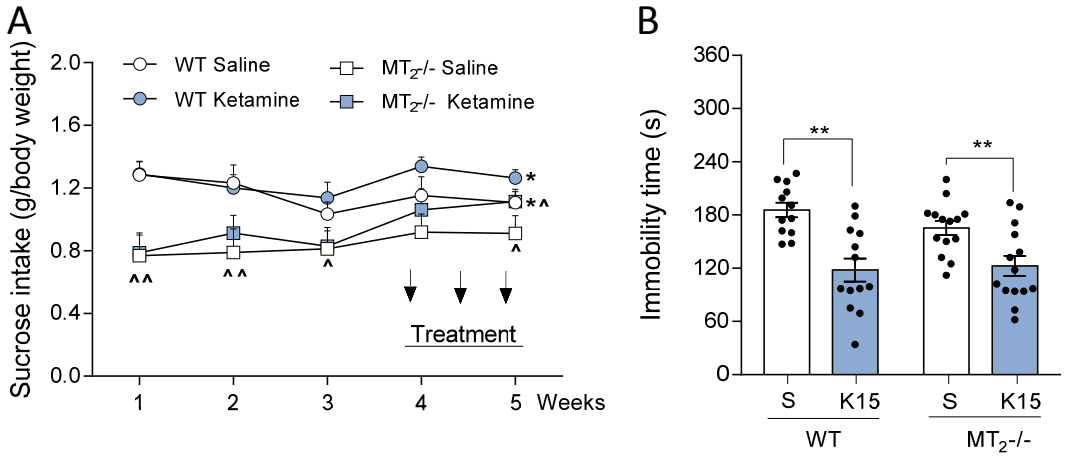

Figure 44. The rapid acting antidepressant ketamine reverses depressive like behavior of MT_2-/- mice. (A) Anhedonic behavior was measured with the sucrose intake test ($F_{1.31}$=5.54 p=0.025) and **(B)** Immobility time (s) on the TST one hour after drug injection ($F_{1.49}$=28.17 p=0.0001). Data show mean ± SEM, n=6-14 mice/group for A and n=12-14 mice/group for B. ***p*<0.01, **p*<0.05; main effect of treatment, ^*p*<0.05; main effect of genotype (Two Way ANOVA).

Discussion

1. The VGLUT1+/- genetic model of depression: behavioral and molecular features

The vesicular glutamate transporters (VGLUT1-3), identified in 2000´s decade, are H^+-dependent carriers that concentrate glutamate into synaptic vesicles, having a key role on synaptic release, plasticity and efficacy of glutamatergic transmission (Wojcik *et al,* 2004). Among the three, VGLUT1 predominates in the cerebral and cerebellar cortices and hippocampus (Vigneault *et al.,* 2015) areas known to play a key role in integrating affective imprints and cognitive processes (Balschun *et al.,* 2010).

VGLUT1 levels modulate depressive-like behavior

This study shows that knock-down of VGLUT1 transporter in rodents impairs both reactivity to rewards and helpless behaviour. Specifically, using a wide population of VGLUT1+/- mice, we show that decreased VGLUT1 levels in the prefrontal cortex (PFC) are linked to anhedonia, a core symptom of depression. Indeed, a wide population of the heterozygous showed a unimodal distribution in the sucrose intake, suggesting that VGLUT1 transporter modulation may be an endogenous mechanism for depressive-like behavioural adjustments. Our lab and others have previously investigated, using small colonies, how a down-regulation of VGLUT1 might influence anxiety, depressive behaviour and learning. Specifically, VGLUT1 heterozygous (VGLUT1+/-) mice, expressing around half cerebral VGLUT1 as do WT, show depressive-like behavior comorbid with mild anxiety and impared recognition memory (Tordera *et al.,* 2007; Elizalde *et al.,* 2010). Matching with our first studies, VGLUT1 knock-down in the mice PFC using lentivirus associated to RNA interference induce depression-like behaviors (Shen *et al.,* 2018; Yu *et al.,* 2018). Further, VGLUT1+/- mice show an increased vulnerability to anhedonia after chronic stress (García-García *et al.,* 2009), suggesting that reduced VGLUT1 negatively affect behavioural outcome in the presence of adverse environmental conditions. In line with all these studies, clinical data show decreased VGLUT1 levels in the PFC of depressed subjects (Uezato *et al.,* 2009; Gilabert-Juan *et al.,* 2012). Further, other preclinical studies support that low VGLUT1 levels could be a biological risk factor of depressive states. Recently, a depression-prone mouse strain created by natural selection of helpless mice showed a downregulation of VGLUT1 in the PFC (Machado *et al.,* 2017). Moreover, different depression models based on chronic stress show VGLUT1 downregulation in cortical areas (Elizalde *et al.,* 2010; Palmfeldt *et al.,* 2016; Wang *et al.,* 2019). Importantly, stress induced VGLUT1 downregulation was more pronounced in females than in males (Shepard & Coutellier, 2018) keeping with the clinical prevalence of this illness.

We observed that VGLUT1 protein levels in the PFC of the heterozygous were slightly upregulated, being 58% of the WT levels. This effect could be caused by feedback mechanisms that either stimulate expression by the single positive allele or provide a better stability of the VGLUT1 mRNA of the heterozygous (Tordera *et al.,* 2007). Interestingly, chronic treatment with the classic antidepressant and noradrenaline reuptake inhibitor reboxetine (15 mg/kg, i.p.) completely rescued PFC VGLUT1 levels in the heterozygous giving evidence that a single allele can also induce VGLUT1 expression up to WT levels when stimulated. Moreover, reboxetine upregulated VGLUT1 in the WT, matching with previous studies in rats where a course of antidepressant treatment consistently upregulated VGLUT1 expression in frontal, orbital, cingulate and parietal cortices, and specific regions of the hippocampus (Tordera *et al.,* 2005; Motsuimilli *et al.,* 2005). These authors have suggested that VGLUT1 upregulation mediated by antidepressants reflects an increase in the number of glutamatergic synapses (Drigues *et al*. 2003; Altar *et al*. 2004; Rapp *et al*. 2004). Importantly, both 5-HT and NA have been linked to the formation and the maintenance of central synapses (Whitaker-Azmitia *et al*., 1995; Matsukawa *et al*., 2003).

Chronic reboxetine restored the anhedonic behavior of VGLUT1+/- mice and reduced helplessness in both genotypes, as previously shown (Palucha-Poniewiera *et al.,* 2017). In agreement with this study, we have previously tested the antidepressant drug imipramine showing similar anti-anhedonic effects to those observed here with reboxetine (Muñoz-Cobo *et al.,* 2018). Reboxetine also reduced natural marble burying behavior, an effect common to all monoaminergic antidepressants (Millan *et al.,* 2001).

On the other hand, systemic administration of the 5-HT_{1A} agonist 8-OH-DPAT induced a lower hypothemic response (Bill *et al.,* 1991) in VGLUT1+/- compared to WT littermates. This result agrees with a previous electrophysiological study carried out in the laboratory of Dr. Lanfumey (París, France) showing that 5-HT_{1A} autoreceptors are desensitized in this model (García-García *et al.,* 2013). 5-HT_{1A} autoreceptors in the dorsal and medial raphe nuclei (DRN and MRN, respectively) exert an inhibitory control on 5-HT neuron firing (Sprouse & Aghajanian, 1987), affecting to 5-HT release in the forebrain. In addition, PFC glutamatergic descending pathways, for which VGLUT1 is the majoritary isoform, innervate 5-HT cell bodies (Celada *et al.,* 2001) or GABAergic interneurons (Hajos *et al.,* 1998; Varga, 2001, Tao & Auerbach, 2003; Amat *et al.,* 2005). It has been suggested that 5-HT_{1A} desensitization in the VGLUT1+/- model could respond to a deficient cortico-raphe glutamate stimulation of 5-HT activity (García-García *et al.,* 2013). In our study, repeated reboxetine treatment rescued the

hypothermic action of 8-OH-DPAT in the VGLUT1+/- mice suggesting that this antidepressant recovers 5-HT_{1A} autoreceptor sensitivity in this model. A previous study has shown that chronic reboxetine does not directly affect to 5-HT_{1A} autoreceptor function (Szabo & Blier, 2001). A possible explanation could be that VGLUT1 upregulation induced by this antidepressant could contribute to reestablish the cortico-raphe glutamate stimulation of 5-HT activity and subsequently, the 5-HT_{1A} function.

These results suggest that VGLUT1, with a key role in glutamate secretion, modulates both depressive-like behaviours and antidepressant action. Previous studies have shown that variations in VGLUT1 levels affect to the glutamate vesicular content and to synaptic availability of glutamate (Wojcik *et al*., 2004; Daniels *et al*., 2004). Thus, VGLUT1+/- mice could address major depression linked to low glutamate in the PFC of depressed patients (Michael *et al*. 2003; Mirza *et al*., 2004; Shirayama *et al.,* 2017; Moriguchi *et al.,* 2018). On the other hand, VGLUT1 upregulation could lead to a rise in glutamate function and initiate a co-ordinated cascade that mediates a change in neural plasticity relevant to antidepressant action (Nibuya *et al.,* 1995; Coppell *et al.,* 2003; Drigues *et al.,* 2003; Altar *et al.,* 2004; Rapp *et al.,* 2004; Melo *et al.,* 2013).

Reboxetine treatment triggers molecular pathways linked to antidepressant action

Several lines of evidence suggest that the effects of monoaminergic antidepressants are linked with an increase in glutamatergic transmission. Firstly, glutamate signalling plays a key role in neuronal plasticity and enhanced glutamatergic transmission induces the expression of neurotrophic factors (Lerea, 1997; Xiao *et al*., 2000; Steward & Worley, 2001). Secondly, strong interactions between monoamines and glutamate systems have been described, like for instance the excitatory effect of 5-HT on cortical glutamate (Aghajanian & Marek, 1997). Especially relevant in this context is the observation that reboxetine that elevates NA in the forebrain, enhances glutamate transmission and synaptic strength in the PFC (Barbon *et al.,* 2011). We next explored in the PFC of WT and VGLUT1+/- mice molecular changes induced by reboxetine linked to glutamate function.

Repeated treatment with reboxetine upregulated the phosphorylated form of GluA1 AMPA subunit receptor (p-GluA1) at Ser831. In addition, this drug induced the expression of the immature form of BDNF (pro-BDNF). Further, the PI3K/Akt/mTOR pathway was activated in both genotypes since it induced the phosphorylation of Akt (pAkt), mTOR (p-mTOR) as well as the two major downstream targets, P70S6 kinase

and p-S6. Thus, as previously observed with SSRIs, chronic reboxetine might activate AMPA receptors in the membrane and consequently initiate a cascade of intracellular signalings that increase protein synthesis and a synaptic strength (Li *et al.,* 2010; Koike *et al.*, 2011; Wolak *et al.*, 2013). Of them, especially relevant for neuronal growth and synaptic connectivity is BDNF (Duman & Monteggia, 2006; Wyneken *et al.,* 2006; Koike *et al.,* 2014; Lipton *et al.,* 2014). In addition, this neurotrophic factor can also activate AMPA dependent glutamate transmission (Shimizu *et al.,* 2003; Slack *et al.,* 2004; Caldeira *et al.,* 2007).

On the other hand, no changes were observed in either of the above proteins in the VGLUT1+/- model suggesting that they are not involved in the depressive-like behavior observed in this model. Yet, these studies should be also performed in other brain regions connected to the PFC.

The VGLUT1+/- model is resistant to the rapid antidepressant action of ketamine

The rapid antidepressant action of ketamine (Fava *et al.,* 2018) was studied in the VGLUT1+/- model. Ketamine at the three doses tested failed to rescue the anhedonic behavior in the VGLUT1+/- mice. In addition, ketamine induced a clear anti-helpless behavior in WT in keeping with previous data (Li *et al.,* 2010) but had no effect in the heterozygous. Moreover, while acute ketamine induced anti-marble burying behavior in WT mice, a common effect observed with antidepressants (Nicolas *et al.,* 2006; Tosta *et al.,* 2018), no effects were seen in VGLUT1+/- mice. These results suggest that ketamine failed to induce in VGLUT1+/- mice the necessary neurochemical mechanisms involved in antidepressant action. So, we hypothesized that deficiencies in synaptic glutamate release are involved in the resistance of this model to ketamine.

We next explored NMDA dependent intracellular pathways following ketamine treatment. A growing number of studies report that the rapid antidepressant response of NMDA receptor targeting drugs, such as ketamine, is due to a transient activation of AMPA receptors (Koike *et al.,* 2011). Downstream AMPA activation, stimulation of the PI3K/Akt/mTOR intracellular pathway induces protein synthesis (Li *et al.,* 2010; Autry *et al.,* 2011; Workman *et al.,* 2013; Dwyer *et al.,* 2015; Yang *et al.,* 2018). In addition, ketamine treatment leads directly to reduced phosphorylation of eEF2 on threonine at position 56 (Thr56) in the cortex enhancing the synthesis of plasticity related proteins (Autry *et al.,* 2011; Kenney *et al.,* 2015; Verpelli *et al.,* 2015). Specifically, reduced p-eEF2 correlates with increased expression of BDNF and p-GluA1 subunit of AMPA

receptors (Duman *et al.,* 2012; Zhou *et al.,* 2014), that, together with other plasticity related proteins enhance synaptic maturation and synaptic strength (Autry *et al.,* 2011).

In our study, p-GluA1/GluA1 and intracellular proteins involved in the PI3K/Akt/mTOR signaling pathway, including p-Akt/Akt and p-mTOR, were upregulated by ketamine in the PFC of WT and VGLUT1+/- mice. Yet, ketamine failed to induce pro-BDNF expression in the VGLUT1+/- model whereas it upregulated pro-BDNF in the WT littermates. Given that BDNF expression has been presented as an essencial protein involved in the rapid antidepressant action of this drug (Autry *et al.,* 2011), our result could explain the observed resistance of the VGLUT1+/- model. Matching with this, ketamine enhanced the inactive phosphorylated form of this factor p-eEF2 in the heterozygous, which would inhibit BDNF synthesis. Conversely, this drug enhanced the active dephosphorylated form of the eEF2 factor in the WTs, which would stimulate protein synthesis and consequently, antidepressant effect. On the other hand, we also observed that the phospho-ERK protein was not activated by ketamine in the VGLUT1+/- mice suggesting that this pathway could be also involved in the drug resistance of this model (Slack *et al.,* 2004; Kenney *et al.,* 2015; Lepack *et al.,* 2016).

Electrophysiological study of the oscillatory activity in the IL cortex of the VGLUT1+/- model revealed higher intensity but lower frequencies of theta band. Interestingly, theta has been associated with cognitive deficit in other mice models (Hölscher *et al.,* 2005, Gong *et al.,* 2009). The observed effects of ketamine (10 mg/kg, i.p.) in delta, theta and gamma oscillations are in agreement with previous studies (Skoblenick *et al.,* 2016, Kohtala *et al*., 2019). In addition, ketamine decreased HFO frequency and increased intensity in the FrA of the WT but not in the heterozygous which, somehow, could contribute to explain the lack of antidepressant effect of ketamine in this model. Importantly, these effects were observed within the first 12 minutes from administration, which, would go in line with the rapid activation of the eEF2k pathway (Zhang *et al.,* 2016). Thus, it is suggested that in these first minutes following ketamine administration it could be decided the beneficial action of this drug.

Rescue of the rapid antidepressant action of ketamine in VGLUT1+/- mice

We further studied how VGLUT1 levels could be involved in the lack of antidepressant effect of ketamine in the heterozygous mice. Therefore two experiments were designed directed to upregulate VGLUT1 in the PFC and then, the antidepressant-like activity of acute ketamine was tested again.

Our first approach was to restore VGLUT1 levels in PFC with the classic antidepressant reboxetine as a priming experiment. Priming has been very useful in order to combine different mechanisms of action (Melo *et al.,* 2013). Combination of classic and rapid antidepressant can be a promising therapeutic approach for those who suffer for MDD with suicidal ideations. After three weeks of treatment, VGLUT1 levels in the PFC and anhedonic behavior were rescued to normal levels as previous experiments. Then after 72 hours of washed reboxetine, ketamine reduce the helpless behavior in the resistant mice VGLUT1+/- whereas washed saline treated mice with ketamine do not reduce his helpless behavior.

Importantly, a single dose of ketamine was enough to dephosphorylate eEF2, suggesting that following repeated reboxetine, ketamine is now able to induce protein synthesis though this pathway in the VGLUT1+/- model. This result also supports a previous study suggesting an essential role of the eEF2k pathway in the antidepressant action of ketamine (Addaikan *et al.,* 2017).

Our second approach was to rescue VGLUT1 levels specifically in the PFC using adeno-associated virus (AAV) technology. In addition we also tested the effect of induced expression of a VGLUT1 mutant (sVGLUT1) in the PFC. This mutated form lacks the glutamate transport function but maintains the VGLUT1 trafficking function in the synaptic vesicles super-pool located in the axons (Herzog *et al.,* 2011).

As expected, our imaging studies confirmed VGLUT1^{mCherryminisog} and sVGLUT1^{mCherryminisog} expression in both cell bodies and fibbers of the PFC. Moreover, no differences between genotypes in the amount of fluorescence (signal area * mean intensity) were detected suggesting that reduced VGLUT1 levels in the heterozygous does not affect to the efficiency of the AAV infection. Interestingly, the expression of VGLUT1^{mCherryminisog} in the PFC was confirmed by western-blot using a specific homemade antibody (Herzog *et al.,* 2011) that recognized both endogenous VGLUT1 (~60 kDa) and VGLUT1 tagged to a fluorescent protein (~100 kDa).

VGLUT1^{mCherryminisog} expression induced an anti-anhedonic action in both genotypes from the second week and was maintained throughout the experiment. However, the expression of the glutamate transport-deficient mutant form, sVGLUT1^{mCherryminisog}, did not affect to the anhedonic behaviour of VGLUT1+/- mice. Interestingly, while acute ketamine exerted an antidepressant-like activity in the VGLUT1^{mCherryminisog} injected VGLUT1+/- mice, it failed to rescue helpless behavior in the sVGLUT1^{mCherryminisog}

injected heterozygous. These results suggest that vesicular VGLUT1 levels can directly modulate the rapid-antidepressant action of ketamine.

It is also relevant to comment that despite these mice were born with reduced levels of VGLUT1 in the brain, overexpression of this transporter in adulthood was sufficient to induce antidepressant-like action. VGLUT1 upregulation in the PFC could enhance synaptic glutamate release and stimulate AMPA-dependent glutamate transmission (Freudenberg *et al.,* 2015). Yet another possibility is that VGLUT1 overexpression in innervated areas from the PFC such as the DRN could enhance locally glutamate release and activate 5-HT firing (Celada *et al.,* 2001; Hajós *et al.,* 1998; Varga, 2001; Tao & Auerbach, 2003).

In line with our study, a previous study has shown that VGLUT1 knock-down in the PFC prevented the rapid antidepressant-like actions of scopolamine. In this study, it is also suggested that VGLUT1-mediated glutamate release and membrane GluA1 activation may play a critical role in the rapid-acting antidepressant-like effects of scopolamine in mice (Yu *et al.,* 2018). Recent mechanistic studies in rodent models have demonstrated similarities in the downstream cellular actions underlying the rapid antidepressant effects of scopolamine and ketamine (Duman *et al.,* 2016, Wohleb *et al.,* 2017, Hare *et al.,* 2017). Notably, scopolamine increases synaptic glutamate and AMPA receptor activation in rodent models (Voleti *et al.,* 2013, Chowdhury *et al.,* 2017), suggesting that the surge in glutamate release is also involved in the rapid-acting antidepressant-like action of scopolamine.

In summary, the present study provides evidence that the vesicular glutamate transporter, VGLUT1, a key gene involved in the regulation of glutamate secretion, modulates depressive-like behavior and antidepressant action. This finding is relevant to preclinical and clinical evidence that increased glutamate underpins neuroadaptive responses induced by both classic and rapid acting antidepressants. Yet, further studies should investigate the postsynaptic mechanisms for the lack of action of ketamine in the VGLUT1+/- mice. Taking into account the hypothesis described by Zanos & Gould (2018) we could suggest that the heterozygous have different postsynaptic glutamate receptor sensitivity. For instance, a higher sensitivity of NMDA receptors to synaptic glutamate release following ketamine treatment could trigger the activation of the eEF2 kinase that would lead to an increase in p-eEF2 and inhibit protein synthesis. Alternatively, these receptors could be less sensitive to the action of ketamine.

2. The melatonin 2 receptor knock-out model of depression: behavioral features, circadian temperature rhythm and antidepressant action

Depressive-like behavior and altered circadian temperature of the MT_2-/- mice.

This study shows that deletion of MT_2 receptors in rodents impairs regulation of both reward sensitivity and circadian temperature rhythm. In particular, MT_2-/- mice exhibit anhedonia, a core symptom of depression (McGlinchey *et al.,* 2006) and a lower amplitude of the circadian CBT compared to WT littermates, which, could be linked to anhedonic behavior (Hasler *et al.,* 2010; Hickie & Rogers, 2011; Moraes *et al.,* 2013). In keeping with this, a link between depressive behaviors and circadian rhythm disruption in animal models has been shown (Logan *et al.,* 2015). Further, a previous study has shown that deletion of MT_2 receptors induced anhedonia (Liu *et al.,* 2017). In addition, pharmacological studies support the involvement of this receptor in circadian clock (Duvocovich *et al.,* 1998; Hunt *et al.,* 2001).

We also studied how reward exposure affected nocturnal CBT in WT and MT_2-/- mice during the mouse active period. During a night of reward exposure, the body temperature of WT was higher than a night under standard conditions. We suggest here for the first time that a positive mean temperature difference between a rewarding and a standard night could be an index of “natural sensitivity for reward”. In keeping with this idea, MT_2-/- mice showed a mean temperature difference close to zero being indicative of decreased reward sensitivity or anhedonia. These studies show that the lack of the melatoninergic receptor MT_2 hampers the rewarding effects of sucrose. Noteworthly, unpublished observations have shown that increase of nocturnal CBT during reward exposure correlates with locomotor activity in WT mice. MT_2 receptors have been reported to be involved in the control of body temperature by melatonin (Fisher & Sugden, 2009; López-Canul *et al.,* 2019) either in synergy with the MT_1 receptor or in an opposite way. The absence of body temperature increase after reward in mice lacking the MT_2 receptor might be due to the deletion of this receptor and the perturbation of the effect of endogenous melatonin in response to reward.

Moreover, MT_2-/- mice showed enhanced vulnerability to stress-induced social avoidance in keeping with other depression models (Venzala *et al.,* 2012) and decreased novelty seeking behavior as previously observed using palatable food (Clough *et al.,* 2018).

Effect of the antidepressants in depressive-like phenotype of MT_2-/- mice.

After chronic treatment, reboxetine (15 mg/kg, i.p.) reverted anhedonia in the sucrose intake test and raised the circadian CBT achieving "a healthy circadian temperature rhythm" in the MT_2-/- genotype. Interestingly, repeated reboxetine treatment raised mean temperature differences between a rewarding and a standard night leading MT_2-/- mice to a "healthy reward sensitivity". Further, reboxetine induced a clear anti-helpless behavior in WT and MT_2-/- mice, in keeping with previous data (Palucha-Poniewiera *et al.,* 2017). However, it failed to rescue novel object place conditioning in MT_2-/- mice suggesting that novelty seeking behavior is unrelated to anhedonic behaviour. In agreement with this hypothesis, impaired novelty seeking behavior in depression models has been interpreted as lack of interest or attention to novel environmental events in depressed patients that could be linked to deficient working memory (Wagner *et al.,* 2006; Gärtner *et al.,* 2018).

On the other hand, systemic administration of the 5-HT_{1A} agonist 8-OH-DPAT induced a lower hypothermic response in the MT_2-/- model compared to WT littermates which would be indicative of 5-HT_{1A} autoreceptor desensitization (Bill *et al.,* 1991; García-García *et al.,* 2013). In addition, reboxetine rescued 5-HT_{1A} sensitivity. The sensitivity of the inhibitory 5-HT_{1A} autoreceptors on cell bodies of serotoninergic neurons has a key role on 5-HT release and mood (Stauderman *et al.,* 1985; Sprouse *et al.,* 1987; Richardson-Jones *et al.,* 2010). Nevertheless since no link between 5-HT_{1A} function and melatonin exist (Hanoun *et al.,* 2004), this observation could be a compensatory mechanism, commonly observed in different animal models of depression (Garcia-Garcia *et al.,* 2009; Amilhon *et al.,* 2010). Altogether, these results suggest that antidepressant efficacy of reboxetine in MT_2-/- mice might be achieved by melatonin independent compensatory mechanisms linked to elevation of monoamine brain levels in key brain areas. Further, our results strength the validity of this genetic model of depression (Willner & Mitchell, 2002).

Finally, the rapid antidepressant action of ketamine (Fava *et al.,* 2018) was also studied in the MT_2-/- model. As expected, ketamine showed a clear anti-anhedonic and anti-helpless action, which further support the validity of the MT_2-/- model (Orozco-Solis *et al.,* 2017). In summary, our study suggests that MT_2 receptors might play a key role in depressive disorders associated to circadian rhythm disruption. Further, this model might be valid to study new antidepressant drugs acting through activation of glutamate function.

Conclussions

1. Mice heterozygous for the vesicular glutamate transporter 1 (VGLUT1+/-) could be considered a genetic model of depression. Study of a wide population of VGLUT1+/- mice revealed that decreased VGLUT1 transporter levels in the PFC are linked to anhedonia, a core symptom of depression. In addition these mice showed helpless behavior and impaired 5-HT_{1A} function. Chronic treatment with the antidepressant reboxetine rescued VGLUT1 levels in the PFC of the heterozygous as well as depressive-like behavior.

2. Repeated treatment with reboxetine induces the expression of the immature form of the brain derived neurotrophic factor (pro-BDNF). In addition, it activates AMPA receptors and subsequently the mammalian target of rapamycin PI3K/Akt/mTOR intracellular signaling pathway. Altogether, these effects might contribute to increase protein synthesis and synaptic strength.

3. The VGLUT1+/- depression model is resistant to the rapid antidepressant action of ketamine. Ketamine failed to rescue the anhedonic behavior of VGLUT1+/- mice and had no effect in either helpless or marble burying behavior. Unlike WT mice, ketamine enhanced the inactive phosphorylated form of the eukaryote elongation factor 2 (p-eEF2) and failed to induce p-ERK expression in the heterozygous, which would inhibit BDNF synthesis. In line with these molecular mechanisms, ketamine did not alter HFO intensities in the VGLUT1+/- mice. We suggest that these molecular and electrophysiological observations, that reflect an impaired glutamate function, might inhibit the antidepressant effects of this drug.

4. Reboxetine pretreatment has a priming effect rescuing the rapid antidepressant action of ketamine in the VGLUT1+/- model. In line with this, ketamine enhanced the active dephosphorylated form of the eukaryote elongation factor 2 (eEF2) in the heterozygous, which would facilitate protein synthesis and rapid antidepressant effects.

5. PFC VGLUT1^{mCherryminisog} expression induced an anti-anhedonic action in both WT and VGLUT1+/- mice. However, the glutamate transport-deficient mutant, sVGLUT1^{mCherryminisog}, had no effect. Moreover, PFC VGLUT1^{mCherryminisog} expression rescued the antidepressant-like activity of ketamine in the heterozygous. These results suggest that vesicular VGLUT1 levels can directly modulate the rapid-antidepressant action of ketamine.

6. MT_2-/- mice exhibit anhedonia and lower amplitude of the circadian CBT compared to WT littermates, which, could be linked to anhedonic behavior. Moreover MT_2-/- mice showed enhanced vulnerability to stress-induced social avoidance and decreased

novelty seeking behavior. Chronic treatment with the antidepressant reboxetine rescued the depressive-like phenotype of MT_2-/- mice. Finally, the rapid antidepressant action of ketamine was confirmed in this model.

7. Here we have studied the effect of reboxetine and ketamine in two genetic models of impaired glutamate and melatonin function. The specific endophenotype of the VGLUT1+/- model has provided a better comprehension of the molecular mechanisms involved in antidepressant response or resistance to treatment. Further, the MT_2-/- model could address, specifically, major depression associated to circadian rhythm disruption and has a good predictive validity to test both classic and rapid-acting antidepressants.

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