Universidad de Navarra

Facultad de Medicina

Studies on the role of HDAC5, SIRT2 and VGLUT1 as pharmacological targets involved in antidepressant action

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Pamplona, Octubre 2017

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Memoria presentada por Dª Irene Muñoz-Cobo Orosa para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Farmacología y Toxicología y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 06 de octubre de 2017

Dra. Rosa María Tordera Baviera

Para mis padres y mi tata.

A mi presente.

A mi pasado.

A mi futuro.

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"Observar sin pensar es tan peligroso como pensar sin observar."

Santiago Ramón y Cajal

Quizá sea más poetisa que neurocientífica o me resulta más natural escribir de este modo, aunque no sencillo. Al escribir agradecimientos lo primero que ven mis ojos y siente mi corazón son las palabras AGRADECER y CIMIENTOS. Creo que no hay dos palabras que puedan definir mejor estas líneas.

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que son horas a tu lado
duras, en casa, no en vano
salvándonos los momentos.
Ir y venir, movimiento,
surgir al desvanecernos
preparando cada paso
de sutil enraizamiento.

Literatura velando
el fácil conocimiento
de la ciencia al resurgir
con un nuevo experimento.
Gracias por estar ahí
donde seguiréis creciendo
aquí hoy quiero transmitir
lo adquirido en este tiempo.

ABREVIATTIONS

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5-HT	5-Hydroxytryptamine, serotonin
AcH	Acetylated histone
AAV	Adeno-Associated virus
BDNF	Brain-derived neurotrophic factor
DSM-V	Diagnostic and statistical manual of mental disorders, 5^{th} edition
DRN	Dorsal raphe nucleus
GABA	γ-aminobutyric acid
HDAC	Histone deacetylase
HAT	Histone acetyltransferase
IL-PFC	Infralimbic prefrontal cortex
MAOI	Monoaminoxidase inhibitor
MAO	Monoaminoxidase
MDD	Major depression disorder
MRN	Medial raphe nucleus
NA	Noradrenaline
NAD	Nicotinamide adenine dinucleotide
NMDA	N-methyl-d-aspartate
PFC	Prefrontal cortex
p-HDAC5	Phospho-histone deacetylase 5

RT-PCR	Real-Time PCR
SIRT	Sirtuin
SNRI	Selective noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
Syn	Synapsin
TLDA	Taqman low density array
VGLUT	Vesicular glutamate transporter
WT	Wild type
YFP	Yellow fluorescence protein

Abstract

Depression is a chronic disorder characterized mainly by depressive mood and anhedonia. The monoaminergic hypothesis has resulted deficient to explain the biological changes that may be caused in the depressive brain. Other hypotheses like alterations in the neuroplasticity, in glutamate transmission, as well as in epigenetics have been proposed. Antidepressant action has been linked to increased synaptic plasticity in which epigenetic mechanisms such as histone posttranslational acetylation could be involved. Interestingly, previous studies in our laboratory have shown that the histone deacetylases HDAC5 and SIRT2 are oppositely regulated by stress and antidepressants in mice prefrontal cortex (PFC). Besides, the neuroblastoma SH-SY5Y line is an *in vitro* neuronal model reliable to study drug effects with clear advantages over animal models. This study focuses on the possible role of two selected epigenetic targets (histone deacetylase 5, HDAC5, and sirtuin 2, SIRT2) and one glutamate target (vesicular glutamate transporter 1, VGLUT1) in antidepressant action.

The first aim was to further explore the role of HDAC5 and SIRT2 in the molecular mechanisms of antidepressants using the *in vitro* SH-SY5Y cellular model. This study shows that nucleocytoplasmic export of HDAC5 and SIRT2 downregulation mediated by antidepressants could enhance synaptic plasticity markers leading to antidepressant action.

The second aim of this study was to evaluate the therapeutic potential of SIRT2 for depression treatment. A course of treatment with the selective SIRT2 inhibitor 33i reversed anhedonia in mice heterozygous for the vesicular glutamate transporter 1 (VGLUT1+/-), considered a genetic model of depression.

In parallel, we initiated a study directed to examine the role of the glutamate target VGLUT1 in the long-loop mechanisms of control of 5-HT activity as well as in the modulation of depressive-like behaviors. Using the adeno-associated virus (AAV) technology, VGLUT1 expression was induced in the PFC of VGLUT1+/-. Interestingly, these preliminary studies have evidenced that VGLUT1 expression in the PFC of VGLUT1+/- mice is linked to antidepressant action.

Altogether, HDAC5 and SIRT2 as well as the synaptic plasticity marker VGLUT1 could be proposed as pharmacological targets involved in antidepressant action.

CONTENTS

INTRODUCTION

1. What is major depression?

Major depression disorder (MDD) is a primary mood disorder characterized by episodes of extreme low mood and loss of interest or inability to experience pleasure in usually pleasant activities, a phenomenon called anhedonia (Skolnick and Basile 2007; Bromet *et al*. 2011). In addition to these core symptoms, other characteristic symptoms that may appear are sleep disturbances, fatigue, futility of guilt, low self-esteem, changes in body weight, negative thoughts with suicidal tendencies and cognitive deficit (Wong and Licinio 2001). According to the Diagnostic and Statistical Manual of Mental Disorders (DMS-V) of the American Psychiatric Association, the diagnoses of MDD is established when the patient presents 3-4 of these symptoms together with one of the core symptoms and persist for at least two weeks.

MDD is one of the leading causes of disability worldwide and has a strong impact both on the daily lives of the patients as well as on the socioeconomic costs to the society. It is the second cause of disability in the world being today the first in industrialized countries. At a global level, MDD affects about 350 million people, an equivalent to 4.7% of the world's population and the number of patients is expected to be increased in the next years. Although MDD affects people of all ages, from all ways of life, the risk of becoming depressed is increased by poverty, unemployment, problems caused by alcohol and drug use, physical illnesses and other stressful life events. Depression is more common among women than men. The World Health Organization declares that depression is one of the main causes of disability, considering itself the most expensive mental disorder in economic terms in Europe, since it represents 33% of the total cost for mental health.

MDD can be classified according to different criteria. Firstly, it can be classified as primary depression when it is not associated to an affective disorder and secondary depression when it is associated to a previous medical or psychological altered condition. 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. Whereas depressive symptoms have a mild but prolonged intensity lasting longer than two years, it is classified as dysthymic disorder. Depressive symptoms may occasionally

appear in certain periods of the year, thus is described as seasonal affective disorder. Adaptive disorders include mild depressive symptoms in which anxiety is produced by identifiable psychosocial factors. Finally, ethologically MDD may be endogenous or reactive depending on whether it has been triggered by external stressors or not.

Among the risk factors, MDD is believed to be caused by the interaction of environmental, social, genetic and epigenetic factors that provoke changes at the biochemical, cytoarchitectural and functional levels in specific areas of the brain (Mazure *et al*. 2000; Belmaker 2008; Krishnan and Nestler 2008).

2. Pharmacological treatment of major depression

According to the classical monoaminergic hypothesis of depression, pharmacological treatment of depression is based on restoring levels of the neurotransmitters serotonin (5-HT) and/ or noradrenaline (NA) that are usually deficient in the MDD (Schildkraut 1965).

First antidepressants discovered in the 1960s include the tricyclic antidepressants (for instance imipramine, amitriptyline or clomipramine), which block the reuptake of biogenic amines, NA and 5-HT, and the monoamine oxidase inhibitors (MAOIs) (like phenelzine), that inhibit the enzyme responsible for the amine degradation, the monoamine oxidase (MAO). However, the frequency of side effects linked to the use of tricyclics or the hypertensive crises produced by MAOIs in interaction with tyramine-rich foods, as well as their inefficacy in a significant percentage of patients, led to the development of new drugs.

Thus, in the 1980s, selective serotonin reuptake inhibitors (SSRIs) (for instance fluoxetine, sertraline, paroxetine or citalopram) emerged and became highly relevant because of their lack of cholinergic adverse effects present in tricyclic antidepressants, apart from being safer drugs.

Other more recent pharmacological drugs include selective noradrenaline reuptake inhibitors (SNRIs) such as reboxetine and inhibitors of both 5-HT and NA transporters such as venlafaxine or duloxetine. Other clinical efficient drugs combine

the action of monoamine reuptake inhibitors and modulators of different receptors such as mirtazapine (5-HT_{2A} and α_2-adrenergic receptor antagonists), or trazodone (5-HT_{2A} antagonist). Finally, the dopamine and NA reuptake inhibitor bupropion has also shown a broad antidepressant action. Still, all these compounds have not been able to improve either the efficacy of classic antidepressants or the latency time of several weeks to obtain a clinical effect (Blier 2003; Machado-Vieira *et al*. 2010).

In the last decade, innovative antidepressant treatments have been proposed. For instance, agomelatine is a melatoninergic agonist that activates the melatonin (MT) receptors MT_1 and MT_2, and restores circadian rhythms, which are usually altered during the course of depression. 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 and Boutin 2006; Kennedy *et al*. 2008; Jockers *et al*. 2008; Guardiola-Lemaitre *et al*. 2014).

On the other hand, recent studies have highlighted the importance of glutamate as a target for the development of new antidepressant drugs, particularly for those cases of treatment-resistant depression (Diazgranados *et al*. 2010; Ibrahim *et al*. 2011). Ketamine is an N-methyl-d-aspartate (NMDA) receptor antagonist. It is currently approved by the US Food and Drug Administration as an anaesthetic agent and it is also used as an off-label medication for the management of chronic pain (Wan *et al*. 2015). In the last year, FDA data support ketamine as a possible depression therapy and in agreement, recent studies have evidenced that ketamine shows rapid and strong antidepressant effects in humans and animal models. However, the use of ketamine has some drawbacks such as its potential abuse and neurotoxicity after chronic treatment (Chaki and Fukumoto 2015). In the last years, new antagonists of NMDA receptors drugs have been proposed for depression treatment. However, these drugs are still following a clinical trial, for instance, AZD 6765 (Astra Zeneca) is in phase II and Delucemine (NPS Pharmaceuticals) is in phase I.

3. Neurobiology of major 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), the difficulty to study pathological changes in the human brain and the limitations of post-mortem brain studies (Frewen *et al.* 2008).

First investigations hypothesized that the development of the disease is linked to an imbalance in brain monoamines (NA, 5-HT, and dopamine). On the other hand, antidepressant action is due to an increase in monoaminergic neurotransmission. Further, it is currently thought that there are other neurotransmitter systems involved such as glutamate and γ-aminobutyric acid (GABA). In addition, a key role of proteins involved in synaptic plasticity like neurotrophic factors and synaptic proteins together with epigenetic mechanisms, genetic polymorphisms or inflammation mechanisms have been proposed. Until now there is no explanation that can unify all these theories, suggesting that MDD could be a heterogeneous disorder. The most relevant theories and hypothesis about the neurobiology of depression are described below.

3.1 The monoaminergic hypothesis of major depression

The monoaminergic hypothesis is the first one postulated on the pathophysiology of MDD. It states that the disorder is caused by the lower availability of monoamines in the brain, mainly 5-HT and NA, and also because their transmission is attenuated (Albert *et al.* 2012). So, the therapeutic strategy is based on administering antidepressant drugs that increase brain levels of 5-HT and NA. This is achieved by inhibiting the reuptake or by inhibiting the degradation of the monoamines to promote monoaminergic transmission (Delgado and Moreno 2000).

However, several findings suggest that it is not possible to explain the development of depression through biogenic amine deficiency exclusively. Firstly, traditional antidepressants are effective only in approximately 60% of patients (Schatzberg 2000) and their therapeutic effect does not begin within several weeks of treatment, despite the immediate increase in levels of monoamines (Jasiak and Bostwick 2014). Moreover, only 30 to 50% of patients show complete remission

(Arrol *et al*. 2005). On the other hand, more than 20% of patients with MDD do not respond to any type of pharmacological intervention (Labermaier *et al*. 2013). Additionally, drugs that increase monoamine levels such as cocaine or amphetamines do not improve depressive symptoms. Finally, experimental depletion of monoamines produces a moderate worsening of mood in untreated depressed patients, but does not affect healthy controls at all (Charney 1998).

Taking into account these limitations and given the latency time of antidepressant efficacy, current research focuses on the study of the long-term neurochemical adaptations occurring in the brain following chronic antidepressant treatment. In this line it has been proposed that clinical effects following chronic antidepressant treatment could be associated to 5-HT_{1A} autoreceptor desensitization (Artigas *et al*. 1996), increased neuroplasticity (Duman *et al*. 1999), activity of glutamate neurotransmission or epigenetic regulation (Nestler *et al*. 2002).

3.2 Glutamate hypothesis of major depression

The glutamatergic system has emerged in recent years as a key modulator of neuronal activity of various neurotransmitter systems and appears to be involved in synaptic plasticity in several brain structures. Accordingly, the glutamatergic system has been implicated in a variety of behavioural functions, including among others the regulation of emotional states, affective, and cognitive processes (Palmfeldt *et al*. 2016).

3.2.1. NMDA receptors

Alterations in NMDA receptors in the PFC affect plasticity in this area and contribute to the pathophysiology of depression (Petrie *et al*. 2000; Pfleiderer *et al*. 2003; Serafini *et al*. 2013; Shipton and Paulsen 2014). Conversely, a growing number of studies are now reporting that NMDA receptor antagonists including ketamine and various selective GluN2B NMDA subunit antagonists induce a rapid antidepressant response in patients (Skolnick *et al*. 2009; Koike *et al*. 2011). In these studies it has been suggested that the modulation of glutamatergic receptors could facilitate neuroplasticity and the release of neurotransmitters such as glutamate and monoamines in the prefrontal cortex (PFC) leading to antidepressant action.

3.2.2. The vesicular glutamate transporter 1

The vesicular glutamate transporters (VGLUT1-3), identified in 2000´s decade, are H^+-dependent carriers that concentrate glutamate into synaptic vesicles, and have a key role on synaptic release and efficacy of glutamatergic transmission (Wojcik *et al*. 2004). Of these, VGLUT1 is the predominant isoform that accounts for most of excitatory glutamatergic terminals in the cortex and hippocampus (Fremeau *et al*. 2001; Herzog *et al*. 2001; Takamori *et al*. 2001; Gras *et al*. 2002; Varoqui *et al*. 2002).

VGLUT1 and VGLUT2 are the predominant isoforms and are highly homologous, expressing exclusively in excitatory glutamatergic terminals and displaying complementary expression patterns. VGLUT1 predominates in the cerebral and cerebellar cortices and hippocampus, whereas VGLUT2 is widely expressed in the diencephalon, brainstem and spinal cord (Fremeau *et al*. 2001; Herzog *et al*. 2001). Furthermore, there is a developmental switch from VGLUT2 to VGLUT1 at P14 (Miyazaki *et al*. 2003; Fremeau *et al*. 2004). The third isoform, VGLUT3, defines a discrete subpopulation of neurons and is co-expressed with cholinergic, serotonergic or GABAergic markers (Schäfer *et al*. 2002; Herzog *et al*. 2004).

Genetic inactivation of VGLUT1 drastically reduces glutamatergic neurotransmission in cortical and hippocampal neurons (Fremeau *et al*. 2004; Wojcik *et al*. 2004). Meanwhile, overexpression of VGLUT1 boosts presynaptic quantal size over WT values (Daniels *et al*. 2004; Wojcik *et al*. 2004). Interestingly, VGLUT1-/- mice show a progressive neurological phenotype including blindness, uncoordination, enhanced startle response and increased lethality rate that starts 2-3 weeks after birth, coincident with the developmental switch from VGLUT2 to VGLUT1 in telencephalic areas (Fremeau *et al*. 2004; Wojcik *et al*. 2004).

The segregated distribution of VGLUT1 and VGLUT2 provides an opportunity to distinguish between cortical and subcortical glutamatergic neurons and to specifically study their pathophysiology. Particularly, prefrontal cortex circuits, in which VGLUT1-positive excitatory neurons are central, play a key operational executive role in integrating affective imprints.

Our lab, has investigated how a down-regulation of VGLUT1 transporter might influence anxiety and depressive-like behavior. We firstly showed that VGLUT1-heterozygous mice expressed half the amount of transporter compared to WT. In addition, a reduction in the reserve pool of synaptic vesicles of hippocampal excitatory terminals and a 35-45% reduction in GABA in the PFC and the hippocampus were observed in the mutant mice. Moreover these mice exhibited increased anxiety in the light–dark exploration test and depressive-like behavior in the forced swimming test (Tordera *et al*. 2007). Moreover, these mice show an increased vulnerability to anhedonia after chronic stress that can be reverted by antidepressants (Garcia-Garcia *et al*. 2009) suggesting that reduced VGLUT1 could be a potential biological risk factor of MDD. Conversely, a course of antidepressant treatment or electroconvulsive shock upregulates VGLUT1 expression in frontal, orbital, cingulate and parietal cortices, and regions of the hippocampus (Tordera *et al*. 2005; Motsuimilli *et al*. 2005). Finnally, a clinical study (Uezato *et al*. 2009) has linked decreased levels of VGLUT1 in the PFC to depressive-like behaviour.

3.2.3. Prefrontal cortex glutamate modulation of 5-HT activity in raphe

5-HT neurons are the most abundant neurons in the dorsal and medial raphe nuclei (DRN and MRN, respectively) and provide dense 5-HT innervation throughout the forebrain. So far, most attempts to model 5-HT system dysfunctions linked to mood disorders have been focused on genetic manipulation of 5-HT targets (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, serotonin transporter SERT, etc.) (Gingrich and Hen 2001). Yet, findings indicate additional complexity on the regulation of 5-HT activity, specifically in the form of local and long-loop feedback mechanisms in the brainstem involving aminoacid neurotransmitter systems. Particularly, glutamatergic descending pathways from the PFC modulate 5-HT activity in the DRN by stimulating directly 5-HT cell bodies (Celada *et al*. 2001) or indirectly through GABAergic interneurons that inhibit 5-HT release (Hajós *et al*. 1998; Varga 2001; Tao and Auerbach 2003; Amat *et al*. 2005) (Figure 1).

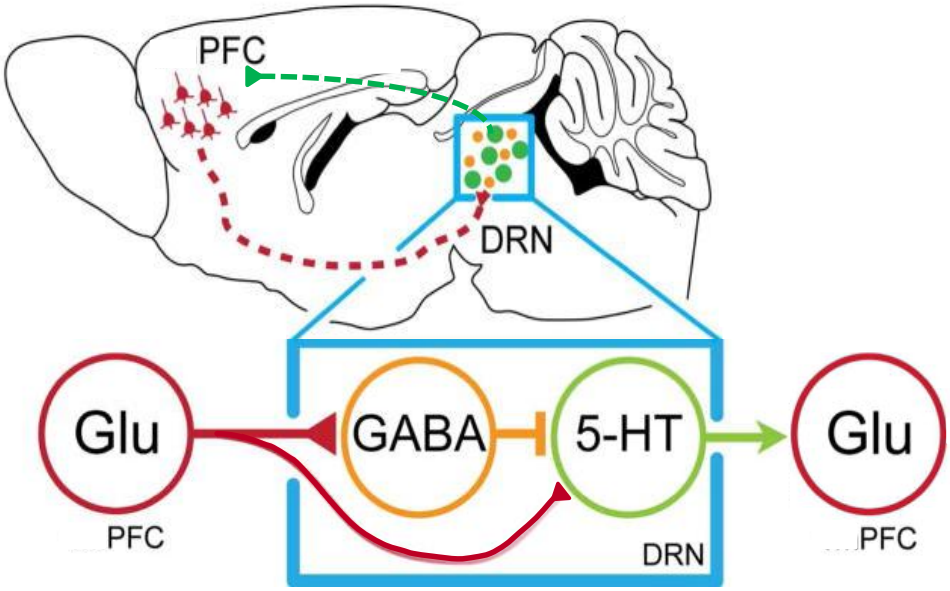


Figure 1. Overview of vmPFC-DRN circuitry. Glutamatergic PFC neurons send afferents to the DRN synapse on GABA interneurons or on 5-HT neurons exerting a long-loop feedback control of inhibitory and excitatory balance on 5-HT activity. Adapted from Challis and Berton 2015.

The PFC has been proposed as the driving structure behind depressive symptoms and antidepressant action due to its role in mood and cognition (Rajkowska 2000). Particularly, deficient PFC glutamate control of 5-HT activity has been linked to depression (Hajós *et al*. 1998; Sharp *et al*. 2007). Conversely, activation of glutamate receptors in PFC stimulates 5-HT release from fibers originated in the raphe nuclei and induces antidepressant action. Moreover, chronic treatment with selective serotonin reuptake inhibitors (SSRIs) enhances both 5-HT release and glutamate synaptic strength in the PFC (Koike *et al*. 2011; Wolak *et al*. 2013).

In this sense, VGLUT1, having a key role on synaptic release and efficacy of glutamatergic transmission in this area (Wojcik *et al*. 2004), could play a key role in the long-loop mechanisms of control of 5-HT system as well as in the modulation of depressive-like behaviors. Particularly, most VGLUT1 positive fibers in the DRN arise from the PFC, where VGLUT1 mRNA is the predominant isoform (Fremeau *et al*. 2001; Härtig *et al*. 2003). A study carried out in our lab suggested that decreased VGLUT1 levels in axon terminals in the DRN could limit synaptic glutamate transmission and affect the excitatory feedback control on 5-HT activity. Specifically, VGLUT1+/- mice,

expressing half of cerebral VGLUT1 compared to wild type (WT) littermates, show decreased 5-HT neuronal activity and 5-HT_{1A} autoreceptor desensitization in the DRN (Garcia-Garcia *et al*. 2013). Thus, deficient VGLUT1 in the PFC could affect 5-HT activity and contribute to trigger depressive symptoms.

3.3 GABAergic hypothesis

Beyond the monoamine theory, in recent years, other theories have emerged suggesting that alterations in GABA may contribute to the development of depression (Sanacora *et al*. 2008). GABA is the main inhibitory neurotransmitter in the brain, whereas glutamate is the main excitatory neurotransmitter. A proper balance between glutamate and GABA levels is essential for normal brain function.

GABA deficiency has been proposed as a model of anxiety and depression. GABA levels in plasma and cerebrospinal fluid have been observed to be low in people with depression (Sanacora and Saricicek 2007). In addition, a cortical GABA deficiency has been observed in depressed patients, which could lead to an excitatory-inhibitory imbalance that can conversely be restored by chronic antidepressant treatment (Sanacora *et al*. 2004; Bhagwagar *et al*. 2007).

3.4 Neuroplasticity hypothesis

Neuroplasticity is the ability of the brain to perceive, adapt and respond to both internal and external stimuli. Plasticity is essential for nervous system healing after injury, stroke or other pathologies allowing a functional recovery of the brain. Neuroplasticity processes include synaptic plasticity, cell growth and remodelling and neurogenesis. Alterations in the regulation of these processes may contribute to the development of a variety of neuropsychiatric diseases. It is postulated that depression could be the result of an inability to adapt to adverse environmental factors, resulting from a dysfunction in normal mechanisms of neuroplasticity (Duman *et al*. 1999). Preclinical and clinical studies, in which structural and cellular alterations are observed in depressed patients, support this theory (Duman 2002). Conversely, enhanced

neuronal plasticity, neurogenesis, dendritic branching, synaptogenesis, appears to be a shared mechanism for antidepressants.

It has been said that neurotrophic factors, most notably the brain derived neurotrophic factor (BDNF), has a role in depression. Stress and depression decrease the expression and function of BDNF in PFC and hippocampus and thus not allowing the proper maintenance of synaptic connections. Conversely, antidepressant treatments (SSRIs) increase BDNF (Björkholm and Monteggia 2016).

Patients with major depression have shown decreased levels of BDNF in brain (Altar *et al*. 2009) and serum (Cunha *et al*. 2006; Palomino *et al*. 2006; Sen *et al*. 2008). 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 and Duman 2008). In line with this hypothesis, several studies have shown that BDNF could mediate the therapeutic action of antidepressants (Berton and Nestler 2006; Groves 2007; Martinowich *et al*. 2008). Several studies show that chronic antidepressant treatments, including selective serotonin reuptake inhibitors and electroconvulsive shock, increase the expression of BDNF in the hippocampus in animal models (Russo-Neustadt 2003; Castrén *et al*. 2007). In line with this hypothesis, mice deficient in BDNF, are used as a model of depression. It has been observed that direct infusion of BNDF into the brain produces an antidepressant effect and potentiates the efficacy of antidepressant treatment, while blockade of BDNF signalling does not (Castrén and Rantamäki 2010; Lindholm and Castrén 2014).

3.5 Epigenetics and depression

Growing evidence suggests that epigenetic mechanisms play a key role in neuronal plasticity. Currently, epigenetics is defined as the study of stable and heritable modifications of chromatin that occur without changes in DNA sequence and influence the phenotypic features of living organisms (Riccio 2010). Studies in human depressed subjects and rodent models, both in brain tissue and in blood cells, have reported epigenetic alterations, associated to depressive behaviour (Covington *et al*. 2009; Hobara *et al*. 2010; Bagot *et al*. 2014).

Among the different epigenetic mechanisms, DNA methylation, non-coding RNA expression and histone modification are the most investigated. Chromatin remodelling is a dynamic process that modulates gene expression. It can be in a condensed state, heterochromatin, which avoids gene transcription, or in an open active state, euchromatin, which allows the genes to be transcribed. Histone modifications affecting chromatin happen mainly in the N-terminal of histones 3 and 4. These modifications include acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation (Karlic *et al*. 2010). Among them, the most studied modification is the acetylation of lysine residues, a process carried out by histone acetyltransferases (HAT) and histone deacetylases (HDAC).

HATs are enzymes that acetylate the lysine residues of histones through a transfer of an acetyl group from an acetyl-CoA molecule, which unwind the DNA-histone conformation, allowing the transcription factors interact with DNA and facilitating gene expression. Conversely, HDACs remove acetyl groups from the lysine residues of the N-terminal tail of histones and other proteins. This deacetylation results in a more compact chromatin state and, therefore, a gene silencing (Figure 2).

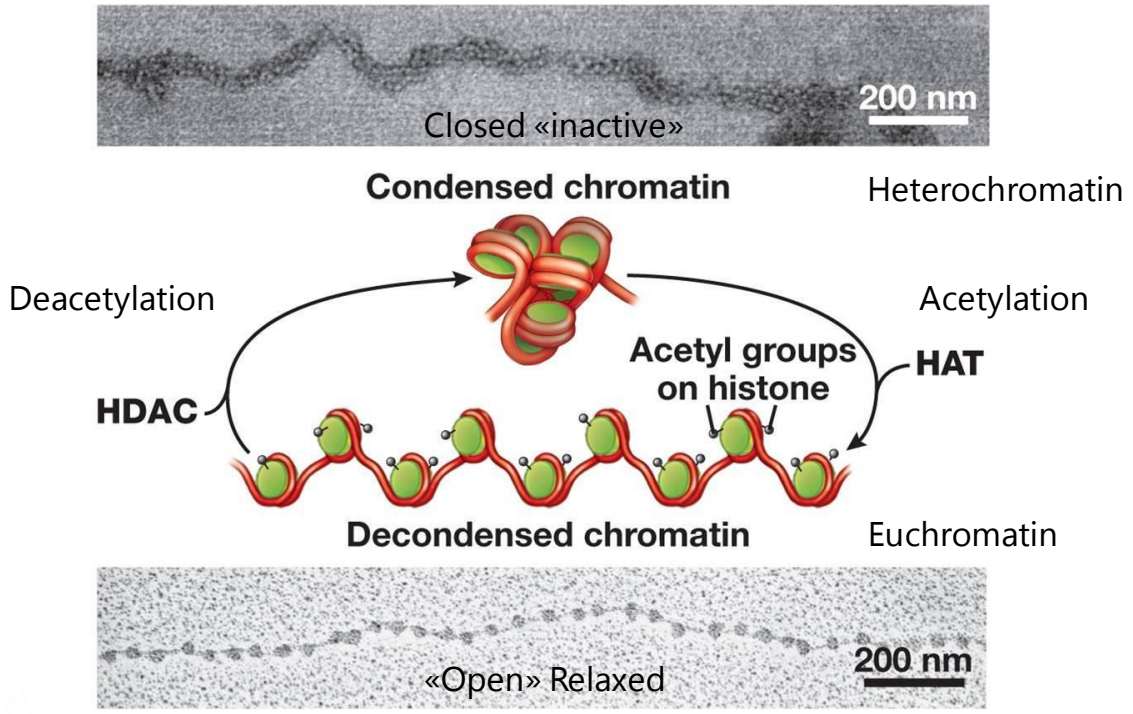


Figure 2. Acetylation and deacetylation of nucleosomal histones play an important role in the modulation of chromatin structure and gene expression. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two opposing classes of enzymes, which tightly control the equilibrium of histone acetylation. Adapted from Pearson Education 2011 (Inc. publishing as Prentice Hall).

The HDACs belong to an evolutionarily conserved family, which is divided into four classes (Haberland *et al.* 2009). Class I, II and IV are similar as they require Zn^{2+} as a cofactor (de Ruijter *et al.* 2003), whereas class III requires nicotinamide adenine dinucleotide (NAD^{+}) (Sauve *et al.* 2006). Class I (HDACs 1, 2, 3 and 8) is found almost exclusively in the nucleus of the cells and is widely expressed in the brain, with the exception of HDAC8 that is muscle specific (Kazantsev and Thompson 2008).

Based on structural parameters, class II is divided into two subclasses: class IIa (HDACs 4, 5, 7 and 9) and class IIb (HDAC 6 and 10). HDAC6 is found predominantly in the cytoplasm, whereas the rest of those subclass II HDACs move between the nucleus and the cytoplasm, through a mechanism regulated by kinase phosphorylation dependent on calcium-calmodulin (Gregoretti *et al.* 2004).

On the other hand, class III HDACs, also called sirtuins (SIRT1-SIRT7) are widely present in several organelles of the cell (Michan *et al.* 2007) and the seven isoforms

have location in the brain (Frye 2000). SIRT1, 2, 6, and 7 are found in the cytoplasm and nucleus, whereas SIRT3, 4 and 5 have mitochondrial localization (Michishita *et al*. 2005).

Class IV consists only of HDAC11. It is mainly found in the nucleus and is expressed during the development of the central nervous system and possibly has a role in inflammation, through its inhibitory effect on the expression of interleukin 10 (Villagra *et al*. 2009).

Changes in histone acetylation have been suggested to play an important role in the pathophysiology of MDD. For instance, clinical studies have confirmed a positive correlation between the expression of some HDACs and the depressive state of patients (Belzeaux *et al*. 2010). Specifically *HDAC2*, *HDAC4* and *HDAC5* mRNA was increased in patients suffering of MDD compared to controls, while mRNA expression of *HDAC6* and *HDAC8* was decreased (Hobara *et al*. 2010). Other clinical study suggests that altered *SIRT1*, *2* and *6* mRNA expression could be associated with the pathophysiology of MDD (Abe *et al*. 2011). Further, non-specific HDAC inhibitors were proved to have antidepressant effects in patients (Machado-Vieira *et al*. 2011). In addition, experimental models based on exposure to stress, have linked upregulation of the histone deacetylases of class I (HDAC1 and HDAC2), class IIa (HDAC4 and HDAC5) and class III (SIRT2) in the PFC and the hippocampus to depressive-like behaviours (Tsankova *et al*. 2006; Renthal *et al*. 2007; Sarkar *et al*. 2014; Erburu *et al*. 2015a). By contrast, specific inhibition of these enzymes were reported to have antidepressant-like activity (Covington *et al*. 2009; Jochems *et al*. 2014; Schroeder *et al*. 2013; Erburu *et al*. 2017). Moreover, repeated monoaminergic antidepressants decrease the levels or the activity of some HDACs (Tsankova *et al*. 2006; Covington *et al*. 2011; Réus *et al*. 2013; Erburu *et al*. 2015a). Thus, HDACs could be promising targets for the treatment of depression (Schroeder *et al*. 2007; Jochems *et al*. 2014).

Recent studies from our laboratory have shown that chronic treatment with antidepressants increased histone 3 and 4 acetylation (AcH3 and AcH4) in mice prefrontal cortex (PFC). In addition in this study we have identified the enzymes HDAC5 and SIRT2, from the class IIa and III HDAC superfamily respectively, as being oppositely regulated by chronic stress and antidepressants (Erburu *et al*. 2015). Thus, decreased

function of these enzymes in the mice PFC might promote histone 3 and 4 acetylation (AcH3 and AcH4) and could play a key role in the mechanisms by which antidepressants enhance synaptic plasticity (Koppel and Timmusk 2013; Erburu *et al*. 2015a).

3.5.1. HDAC5 in depression and antidepressant action

HDAC5 belongs to the class IIa HDAC superfamily and shuttles from the nucleus to the cytoplasm through a phosphorylation mechanism (McKinsey *et al*. 2001; Chawla *et al*. 2003; Renthal *et al*. 2007). The amino-terminal domain of the enzyme is subject to a reversible phosphorylation that controls its nucleo-cytoplasmic distribution. The non-phosphorylated fraction remains in the nucleus, bound to the chromatin and repressing the transcription while the phosphorylated fraction leaves the nucleus, allowing the expression of its target genes (Parra and Verdin 2010).

A relevant study has shown that overexpression of HDAC5 blocks the antidepressant effect of imipramine in the chronic social defeat stress (CSDS) model and this effect is due to the repressive effect of HDAC5 over BDNF expression (Tsankova *et al*. 2006). In agreement with this study, an increase in *HDAC5* mRNA in the cerebral cortex of subjects with MDD compared to control has been observed (Hobara *et al*. 2010). In addition, we have shown in our lab that while the CSDS model upregulates nuclear HDAC5, the tricyclic imipramine and the selective noradrenaline reuptake inhibitor reboxetine increased the phosphorylated form of HDAC5 (p-HDAC5), mainly located in the cytoplasm (Erburu *et al*. 2015a). Interestingly, non-selective inhibition of the class II HDAC superfamily leads to an increase in synaptic plasticity markers (Erburu *et al*. 2015a). Yet, the effect of a selective HDAC5 inhibition on synaptic plasticity has not been evaluated yet.

On the other hand, HDAC5 has been implicated in other physiological functions. For instance, it has been shown that the phosphorylation of HDAC5 in cardiac tissue induces cardiac hypertrophy (Lehmann *et al*. 2014), while in the vascular endothelium it induces angiogenesis (Urbich *et al*. 2008) and also stimulates the expression of anti-inflammatory genes (Wang *et al*. 2010). Finally, recent research associates an increase in nuclear HDAC5 to tumor formation, presenting p-HDAC5 as a possible biomarker of

efficacy in the response to chemotherapy (Kin and Benchimol 2013). Taken together, these findings raise the question of whether depressive state or antidepressant treatment regulates the expression of genes directly associated to HDAC5 function.

3.5.2. SIRT2 in depression and antidepressant action

SIRT2 belongs to the class III NAD^+-dependent histone deacetylases. Due to its participation in metabolic homeostasis and brain aging, sirtuins have aroused a growing interest in neurodegenerative diseases. Among all sirtuins, SIRT2 is the most expressed at the brain level (Pandithage *et al*. 2008). SIRT2 resides in the nucleus, performing its histone deacetylase function, but also, in an important way, in the cytoplasm where it participates in the organization of the cytoskeleton, targeting α-tubulin (North *et al*. 2003). Particularly, a polymorphism of the SIRT2 gene has been related to depression in patients with Alzheimer's disease (Porcelli *et al*. 2013). On the other hand, the inhibition of SIRT2 exerts neuroprotective effects in various models of neurodegenerative diseases, including Parkinson's disease (Outeiro *et al*. 2007) and Huntington (Taylor *et al*. 2011).

Studies in our laboratory have shown an increase in expression of mRNA and protein of SIRT2 in the PFC of mice exposed to CSDS. Regarding the mechanism of action involved in this effect of imipramine, both the selective noradrenaline reuptake inhibitor, reboxetine, and the selective serotonin reuptake inhibitor, fluoxetine, decreased the expression of SIRT2, suggesting that this could be a shared mechanism of monoaminergic antidepressants in general. In the same way these antidepressants increased the acetylation of the cytoskeletal protein α-tubulin, a cytoplasmic SIRT2 substrate (Erburu *et al*. 2015a). Further, the specific inhibition of SIRT2 by the chronic administration of 33i produced an increase in the expression of AcH4, CREB and pro-BDNF in the PFC, suggesting that the inhibition of SIRT2 modulates synaptic plasticity (Erburu *et al*. 2015a; 2017).

Taken together, these results suggest that inhibition of SIRT2 could be another mechanism by which antidepressants stimulate the expression of genes involved in synaptic plasticity. In line with this hypothesis, our results showed an increase in the

expression of *SIRT2* mRNA in the PFC of depressed patients confirming the potential interest of this enzyme for the therapeutic intervention.

3.6 Other hypothesis

There are other hypotheses that are suggested for MDD development. For instance, neuroendocrine hypothesis suggests that MDD is associated with an inadequate stress response due to hypothalamic-pituitary-adrenal axis dysfunction (Holsen *et al*. 2013). Further, increased levels of glucocorticoids affect hippocampal neurogenesis contributing to the development of the disease (Anacker *et al*. 2013). In addition, there is evidence suggesting that corticosteroids modify BDNF function, which would indicate its potential involvement in the pathogenesis of MDD (Kumamaru 2008).

On the other hand, several papers suggest that the immune system is also implicated in MDD (Makhija and Karunakaran 2013). In fact, depressed patients show increased levels of glucocorticoids and proinflammatory cytokines (Liu *et al*. 2012). Proinflammatory cytokines have important metabolic and endocrine effects, including neurotransmitter metabolism, neuroendocrine function and neuronal neuroplasticity.

Other emerging suggested theories have implicated the role or intestinal microbiota that could be influencing normal brain chemistry and, therefore, behaviour (Hoban *et al*. 2016). As a matter of fact, it has been found that bacteria translocation from the intestinal tract to the brain could generate neuroinflamation linked to depressive-like behaviour (Bravo *et al*. 2012; Rogers *et al*. 2016; Martín-Hernández *et al*. 2016). Peptides produced at the gastrointestinal level such as leptin, ghrelin and cholecystokinin (Kluge *et al*. 2011), among others, have a direct influence on the central nervous system, including neurogenesis, which may be involved in depressive disorder (Villanueva 2013).

4. The SH-SY5Y neuroblastoma cell line as an *in vitro* neuronal model: study of the mechanisms of antidepressants

In vitro neuronal cell lines provide a major advantage for the study of new drugs in the central nervous system compared to animal models because of the accessibility of the drugs to the cells and the controllability of many conditions. The neuroblastoma SH-SY5Y cell line is an *in vitro* neuronal model retaining many biochemical and functional properties of neurons (Biedler *et al*. 1978; Ross *et al*. 1983). This cell model has been widely used to study physiology, metabolism, neurodegeneration and neuroadaptation processes in neurons. Interestingly, in this system, a variety of monoaminergic antidepressants have been tested in these cells. For instance, several studies show the induction of BDNF (Donnici *et al*. 2008) and other neurotrophic factors by antidepressants and their neuroprotective effects (Shadfar *et al*. 2016). Moreover, new compounds with a potential antidepressant action have been tested in this cell model (Fukuda *et al*. 2016) together with the exploration of different cell signalling pathways involved in the mechanisms of action of antidepressants (Hu *et al*. 2014).

APPROACH AND OBJECTIVES

Major depression is a mental disorder characterized mainly by extreme depressive mood and anhedonia together with other psychosomatic associated symptoms. This illness can cause moderate or severe disrepair in the patient life, including personal areas. It is turning it into one of the leading causes of disability worldwide. For instance, in Spain, it is estimated that almost 5 million people present mild depressive symptoms while another 1 million show moderate to severe symptomatology. Given that this is a chronic disease, these data reveal a huge social problem in terms of human suffering for the patients and their families, as well as a high economic cost for developed countries.

The monoaminergic deficiency hypothesis has for years been the basis of therapeutic approaches for the treatment of depression. However, this hypothesis has been questioned due to the absence of an immediate efficacy of the treatments, to the failure of these treatments as well as to the numerous relapses that some patients present. Therefore, it is believed that a chain of biological events of greater complexity than the simple alterations of cerebral monoamine levels might take place. Other hypotheses have been proposed, like for instance alterations in neuroplasticity or in glutamate transmission as well as in epigenetics. Yet, at present, there is no common explanation that brings together the different theories.

Recent observations based on the effect of environmental factors on epigenetic targets suggest that biological risk factors of depression could be epigenetic. In addition, such epigenetic modifications could explain the enormous inter-individual variability towards adversity or response to treatment. In this context, current research focuses on the antidepressant regulation of epigenetic targets as well as its role in neuroplasticity.

Antidepressant action has been linked to increased synaptic plasticity in the prefrontal cortex (PFC), in which epigenetic mechanisms could play a key role. Of these, the histone deacetylase (HDACs) enzymes have been proposed as epigenetic targets involved in the pathophysiology of depression and antidepressant-like action. Specifically, previous studies carried out in our lab have identified the class IIa histone deacetylase 5 (HDAC5) and the Class III sirtuin 2 (SIRT2) as being oppositely regulated

by stress and antidepressants. Importantly, decreased function of these enzymes might promote histone acetylation and induce the expression of different plasticity markers highly linked to depression and antidepressant action.

In vitro neuronal cell lines provide a major advantage for the study of new drugs in the central nervous system compared to animal models because of the accessibility of the drugs to the cells and the controllability of many conditions. The neuroblastoma SH-SY5Y cell line is an *in vitro* neuronal model retaining many biochemical and functional properties of neurons. This cell model has been widely used to study physiology, metabolism, neurodegeneration and neuroadaptation processes in neurons. Interestingly, several studies have tested in these cells the effect of antidepressants on neuroplasticity.

Accordingly, in the frame of this project our first aim was to further explore the role of HDAC5 and SIRT2 in the molecular mechanisms of antidepressants. Using the *in vitro* SH-SY5Y cellular model we have studied the antidepressant regulation of these epigenetic targets and their involvement in synaptic plasticity. Specifically, the expression of the synaptic plasticity markers the brain derived neurotrophic factor (BDNF) and the vesicular glutamate transporter 1 (VGLUT1), both highly linked to antidepressant action, have been studied.

Subsequently, our second aim was to study the therapeutic potential of SIRT2 for depression treatment. Specifically, the potential antidepressant effect of the selective SIRT2 inhibitor 33i was tested in mice heterozygous for VGLUT1 (VGLUT1+/-), previously characterized as a genetic model of depressive-like behaviour.

On the other hand, clinical and preclinical studies suggest a key role of the PFC glutamatergic signaling and a therapeutic value for glutamatergic targets in major depression. Particularly, glutamatergic descending pathways from the PFC, in which VGLUT1-positive excitatory neurons are central, modulate 5-HT activity in the dorsal raphe nuclei. Here we have initiated a study directed to examine the role of the PFC VGLUT1 in the long-loop mechanisms of control of 5-HT system as well as in the modulation of depressive-like behaviors. Firstly, an adeno-associated virus (AAV) expressing the VGLUT1 gene, has been designed. Subsequently, using the VGLUT1+/-

model, preliminary studies directed to evaluate the effect of induced VGLUT1 expression in the PFC on depressive-like behaviour as well as on 5-HT_{1A} function have been carried out.

The specific aims of this project are:

1. **To explore the role of HDAC5 and SIRT2 in the molecular mechanisms of antidepressants. Specifically, using the SH-SY5Y cell line we have studied:**

   1.1. The effect of 2 and 24 hours incubation with the antidepressants imipramine, fluoxetine and reboxetine on HDAC5 and SIRT2 expression. In addition, the effect of the selective HDAC5 inhibitor MC3822 and the selective SIRT2 inhibitor 33i on histone acetylation, AcH3 and AcH4, and on the synaptic plasticity markers VGLUT1 and BDNF has been studied.

   1.2. The effect of 2 and 24 hours incubation with the antidepressants imipramine, fluoxetine and reboxetine on these epigenetic targets, histone acetylation and synaptic plasticity markers.

   1.3. The effect of the muscarinic cholinergic receptor antagonist scopolamine on histone acetylation and synaptic plasticity markers.

   1.4. The long-term antidepressant regulation of all these epigenetic and synaptic plasticity markers *in vivo*, in the mice PFC, comparatively to the *in vitro* studies.

2. **To explore the therapeutic potential of SIRT2 for depression treatment in an animal model. Specifically, using the VGLUT1+/- model we have studied whether:**

   2.1. The VGLUT1+/- model shows alterations in the expression of the HDAC superfamily or in histone acetylation in the PFC.

   2.2. SIRT2 inhibition, using the selective inhibitor 33i, reverses anhedonic behaviour in the VGLUT1+/- mice. The antidepressant imipramine was used as a reference compound.

2.3. The selective SIRT2 inhibitor 33i interacts with specific monoaminergic molecular targets for depression treatment such as the serotonin or noradrenaline transporters as well as the monoaminooxidase enzyme.

3. To study the role of induced expression of VGLUT1 in the PFC in antidepressant action. Specifically, using the VGLUT1+/- model, the following studies have been carried out:

3.1. Design of the plasmid pAAV-pSyn-VGLUT1-mCherryminisog-SMD2 and evaluation of its functionality in rat hippocampal cultures. Subsequently, design of the adenovirus pAAV-pSyn-VGLUT1^{mCherryminisog}.

3.2. Determination of the optimal concentration required for imaging VGLUT1^{mCherryminisog} expression both in the PFC and in glutamatergic descending fibbers in the dorsal raphe nucleus (DRN) of VGLUT1+/- and WT littermates.

3.3. Effect of VGLUT1 induced expression in the PFC in the anhedonic-like behaviour of VGLUT1+/- mice compared to WT as well as in a battery of behaviours.

3.4. Effect of VGLUT1 induced expression in the PFC on the hypothermic response mediated by a 5-HT_{1A} agonist as a simple functional approach to evaluate the 5-HT_{1A} autoreceptor sensitivity.

MATERIAL AND METHODS

1. Cell cultures

Human SH-SY5Y neuroblastoma cells (ECACC; Sigma Aldrich, St. Louis, MO, USA; ATCC® CRL-2266™) were cultured at 37°C, in humidified air with 5% CO2, in Dubbelco`s Modified Eagle Medium (DMEM; gibco, Life Technologies, Paisley, UK) supplemented with 10% of fetal bovine serum (FBS; gibco, Life Technologies, Paisley, UK), and Penicillin/Streptomycin solution (Lonza, 17-602E) at 100 U/mL. The medium was changed twice a week and cells were split at about 80% confluence. Cells were grown in tissue culture flask of 75cm^2 (BD Falcon, Franklin Lakes, NJ, USA) and cells were never cultivated beyond passage 25. Cells were seeded in 6 well cell culture clusters (Costar, Corning, NY, USA) at the density of 200,000 cells per well. For each experiment, 6 cell cultured wells per drug treatment or vehicle were used.

2. Animals

Male C57BL/6J mice (Harlan, France, 8 weeks of age, n=8/group) were housed in individual cages and allowed to habituate for 2 weeks before beginning antidepressant treatments.

One male Wistar rat (180–220 g) (Harlan, France) for the affinity for serotonin transporter (SERT) and noradrenaline synaptosomal uptake studies was used.

A colony of heterozygous VGLUT1 (VGLUT1+/-) and wild-type (WT; C57BL/6) male mice (8-10 weeks old, n= 7-9 animals/group for the 33i *in vivo* experiment and 9-11 animals/group for the AAV experiment) were bred in the animal house of the University of Navarra from heterozygous fathers (Dr. S. Wojcik, Gottingen, Germany) (Wojcik *et al*. 2004) and WT mothers (Harlan, France). Mice were weaned and genotyped at the age of three weeks. VGLUT1+/- mice were studied and compared to their WT littermates. Heterozygous mice exhibited no apparent phenotypic abnormalities during development and adulthood.

Food and water were available *ad libitum* for the duration of the experiments. Animals were maintained at a temperature (21±1°C) and humidity-controlled room (55±2%) on a 12 h light-dark cycle (lights on at 08:00 h).

Experimental procedures and animal husbandry were conducted according to the principles of laboratory animal care as detailed in the European Communities Council Directive (2013/53/EC) and approved by the Ethical Committee of University of Navarra.

3. Drugs and treatments

For cell culture experiments the antidepressants imipramine HCl (Sigma Aldrich, USA), fluoxetine HCl (Interchim, Mountluçon, France) and reboxetine HCl (kindly donated by Servier Laboratories, Paris, France) and also the antimuscarinic drug scopolamine HCl (Sigma Aldrich, USA) were dissolved in sterile DMSO to a stock concentration of 1 mM. The selective HDAC4/5 inhibitors MC3822 and MC3823 and the SIRT2 inhibitor 33i were dissolved in sterile DMSO to a stock concentration of 1 mM. MC3822 and MC3823 were kindly donated by Dr Valente (Sapienza University of Rome, Italy). The compound 33i, was kindly donated by Dr Suzuki from Kyoto Prefectural University of Medicine, Japan.

For *in* vivo administration, Imipramine HCl, fluoxetine HCl and reboxetine HCl were dissolved in saline (0.9%). The compound 33i was prepared in suspension using 18% tween 80 and 5% DMSO in saline.

The antidepressant imipramine is a serotonin and noradrenaline reuptake inhibitor that increases the levels of neurotransmitters, noradrenaline and serotonin, in the synaptic cleft. As a tricyclic antidepressant, it has also a weak affinity for muscarinic (M1), histaminic (H1) and adrenergic (α1) receptors. Fluoxetine is, in turn, a selective serotonin reuptake inhibitor whereas reboxetine is a selective noradrenaline reuptake inhibitor.

The compound MC3822 (1R,2R,3R)-N-hydroxy-2-(4-(oxazol-5-yl)phenyl)-3-phenylcyclopropane-1-carboxamide) has been reported as specific HDAC4/5 inhibitor, (Bürli *et al*. 2013) while no information is available for the enantiomer MC3823 (1S,2S,3S)-N-hydroxy-2-(4-(oxazol-5-yl)phenyl)-3-phenylcyclopropane-1-carboxamide). Thus, the corresponding racemate was prepared by Dr Valente team, and the two pure

enantiomers were separated and identified through analytical methods. Afterwards, the two compounds were tested against HDAC1-9 enzymes to assess their inhibition capabilities.

The compound 33i is a 3'-phenethyloxy-2-anilinobenzamide analogue (2-{3-(3-fluorophenethyloxy)phenylamino}benzamide) representing a new class of potent SIRT2-selective inhibitors. In previous studies it has shown IC_{50} of 570 nM towards SIRT2 and no affinity for the rest of sirtuins (Suzuki *et al.* 2012).

4. Experimental design

4.2. Experimental design 1: Role of HDAC5 and SIRT2 in the molecular mechanisms of antidepressant

Cell cultures. Two independent experiments were carried out. In the first experiment, cultured wells were incubated with imipramine, fluoxetine, reboxetine and scopolamine to a final concentration of 10 μM or vehicle containing 1% of dimethyl sulfoxide (DMSO), for 2 and 24 hours at 37ºC. In a second experiment, cells were incubated with the selective HDAC4/5 inhibitors MC3822 and MC3823, the SIRT2 inhibitor 33i (5 μM for 2 and 24 h) or vehicle. Then, cells were collected and epigenetic and synaptic plasticity markers were studied by Western blot and immunofluorescence.

Animal treatments. Mice were randomly divided into four groups and received daily i.p. injections of imipramine (10 mg/kg), fluoxetine (15 mg/kg), reboxetine (15 mg/kg) or saline once a day (at 10 a.m.) for three weeks. A single daily dose was selected in order to minimize the stress due to the injection. At this dose these antidepressants have shown to effectively enhance extracellular levels of noradrenaline and/or serotonin.

Twenty four hours 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 infralimbic 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. Then, Western blot and RT-PCR studies were carried out directed to study the expression of synaptic plasticity and epigenetic targets (Figure 3).

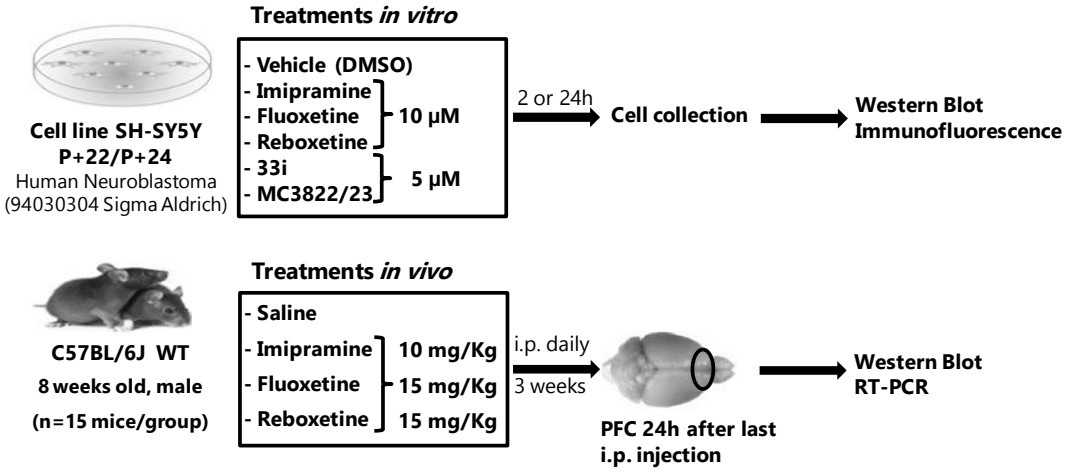


Figure 3. Experimental design 1. Antidepressant regulation of epigenetic targets linked to synaptic plasticity *in vitro* and *in vivo*.

4.2. Experimental design 2: Effect of SIRT2 inhibiton on anhedonic behaviour of the VGLUT1+/- depression model

In a first experiment, mice were divided into saline and imipramine groups. Mice (n= 15-30 mice per group) received daily intra-peritoneal (i.p.) injections of imipramine (10 mg/kg) or saline (at 10 am) for three weeks. In the second experiment, mice were divided into saline and 33i groups. Mice (n= 15-30 mice per group) received daily (i.p.) injections of 33i (15 mg/kg) or saline (at 9 am) for three weeks. Anhedonic-like behavior was analyzed by weekly monitoring of sucrose intake (Figure 4).

In a third experiment no treatment was implemented. We compare in both genotypes the mRNA expression of the enzymes belonging to the histone deacetylase superfamily. Mice were killed by cervical dislocation (2 hours after the last dose, in the case of imipramine or 33i treatment). Brains were rapidly removed and the PFC (around 15 mg) was rapidly 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 infralimbic 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 for neurochemical studies.

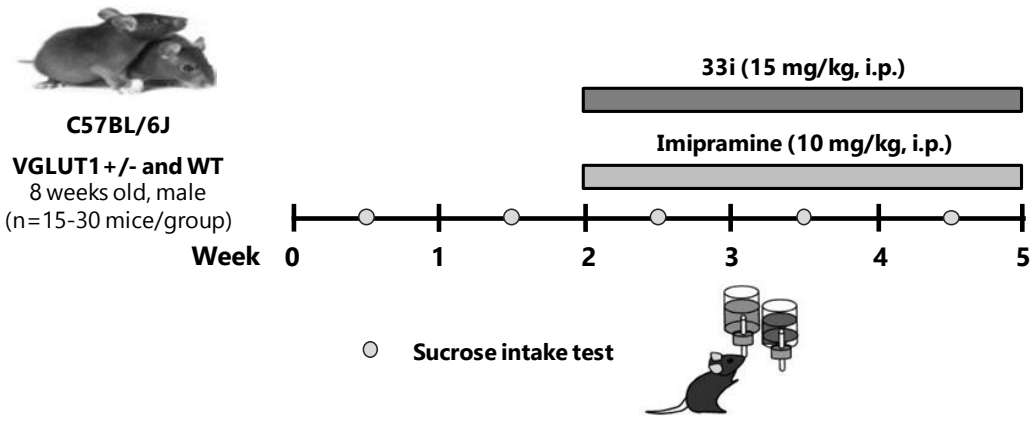


Figure 4. Experimental design 2. Effect of SIRT2 inhibition on anhedonic behaviour of VGLUT1+/- mice.

4.3. Experimental design 3: Effect of VGLUT1-induced expression in the PFC in antidepressant action

Both VGLUT1+/- and WT littermates were divided in two groups of 8-9 mice and received stereotaxic injections of either pAAV-pSyn-VGLUT1^{mCherryminisog} or pAAV-pSyn-YFP in the prefrontal cortex (PFC).

Anhedonic-like behavior was analyzed by monitoring sucrose intake before stereotaxic injections (week 0) and after (weeks 1, 2, 3 and 7). On the fourth and fifth week, a battery of behavioural test was applied in the following order: spontaneous motor activity and open field test (day 1), social interaction test (day 4), marble burying test (day 7). In addition, core body temperature and hypothermic response induced by 8-OH-DPAT was tested on day 10.

Mice were killed by anaesthetic overdose on week 8, perfused with intracardial injection of paraformaldehyde and brains were processed for tissue imaging experiments (Figure 5).

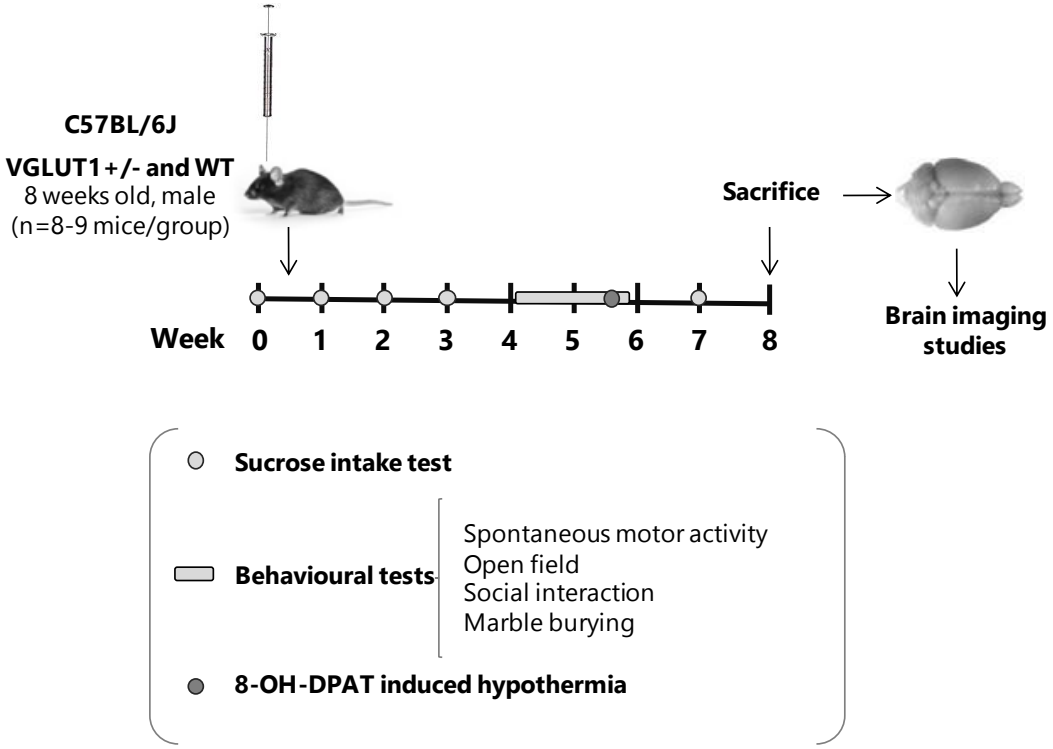


Figure 5. Experimental design 3. Effect of VGLUT1 induced-expression in the PFC and projecting areas in depressive-like behaviour of VGLUT1+/- mice.

5. Enantioseparation of MC3822 and MC3823

The direct HPLC enantioseparation of MC3822 and MC3823 was carried out on the polysaccharide-based Chiralcel OJ-H chiral stationary phase (CSP) using the mixture n-hexane-ethanol-TFA 65:35:0.1 as a mobile phase. In order to identify the enantiomeric elution order, the circular dichroism signal at the single wavelength of 254 nm was monitored during chromatography and compared with that obtained in the enantioselective analysis of MC3822 and MC3823 on the Chiralpak IC CSP under the same elution conditions. As previously reported, using the latter CSP the (S,S,S)-enantiomer (MC3823) was eluted before than the (R,R,R) form (MC3822).

The analysis of the chromatograms obtained by simultaneous on-line CD and UV detection (Figure 6) provides evidence that CD peaks pertinent to the first eluting enantiomers in both enantioselective methods have the same negative sign and, consequently, the same (S,S,S) absolute configuration.

HPLC enantioseparations were performed by using stainless-steel Chiralcel OJ-H (250 mm × 4.6 mm i.d. and 250 mm × 10 mm i.d.) and Chiralpak IC (250 mm × 4.6 mm i.d) columns (Chiral Technologies Europe, Illkirch, France). All chemicals, solvents for HPLC, and syntheses and spectral grade solvents were purchased from Sigma-Aldrich and used without further purification.

The analytical HPLC apparatus consisted of a PerkinElmer 200 LC pump equipped with a Rheodyne injector, a 20 mL sample loop, a HPLC Dionex CC-100 oven and a Jasco Model CD 2095 Plus UV/ CD detector. For semi-preparative separations, a PerkinElmer 200 LC pump equipped with a Rheodyne injector, a 500 mL sample loop, a PerkinElmer LC 101 oven and Waters 484 detector were used. The signal was acquired and processed by the Clarity software of DataApex (Figure 6).

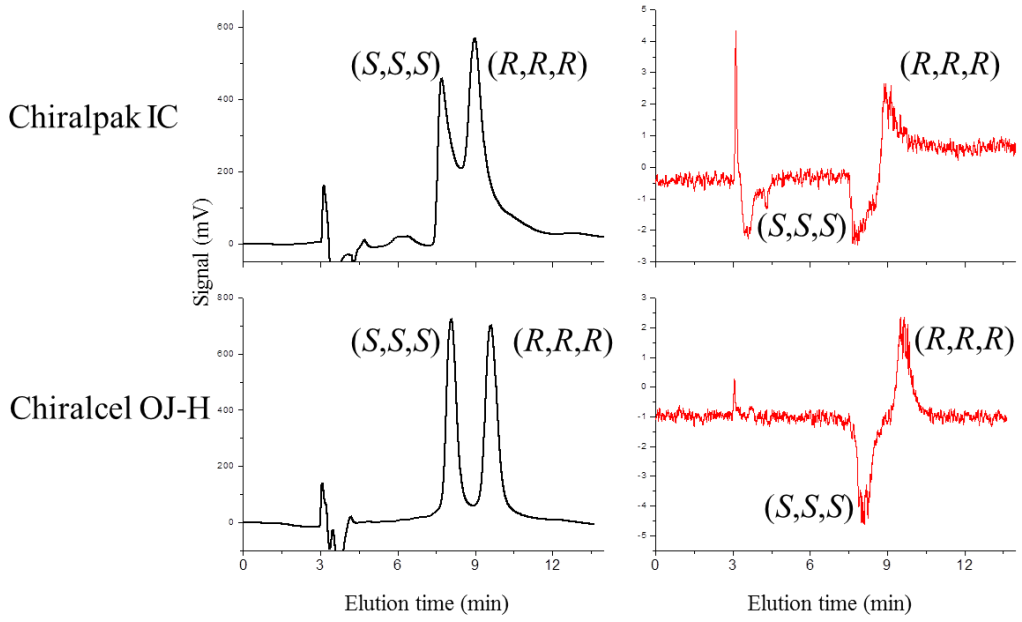


Figure 6. UV (black) and CD (red) chromatograms of compound MC3822 and MC3823.

Columns: Chiralpak IC (top) (250 mm x 4.6 mm i.d.) and Chiralpak OJ-H (bottom) (250 mm x 4.6 mm i.d.); eluent: n-hexane-ethanol-TFA 65:35:0.1; flow rate:1 mL min-1; column temperature: 25°C.

6. HDAC1-9 isoforms inhibition assay

MC3822 and MC3823 were tested in ten-dose IC_{50} mode with threefold serial dilution starting from 200 µM (HDAC1,2,3), from 100 µM (HDAC6,8) and from 50 µM (HDAC4,5,7,9) solutions. Individual IC_{50} values for each HDAC isozyme were measured with the homogeneous fluorescence release HDAC assay. Purified recombinant enzymes were incubated with serial diluted inhibitors at the indicated concentration. The deacetylase activities of HDACs 1-9 were determined by assaying enzyme activity using for HDAC1, 2, 3, 6 the fluorogenic peptide from p53 residues 379-382 (RHKK(Ac)AMC) substrate, for HDAC4, 5, 7, 9 the fluorogenic HDAC Class IIa substrate (Trifluoroacetyl Lysine) and for HDAC 8 the fluorogenic peptide from p53 residues 379-382 (RHK(Ac)K(Ac)AMC) substrate. Deacetylated AMC-substrates were sensitive toward lysine peptidase, and free fluorogenic 4-methylcoumarin-7-amide was generated, which can be excited at 355 nm and observed at 460 nm (Reaction Biology Corporation, MD, USA). Data were analyzed on a plate-to-plate basis in relationship to the control and imported into analytical software (GraphPad Prism, CA, USA).

7. RT-PCR studies

Total RNA extraction. Total RNA of prefrontal cortex (PFC) samples of mice chronically treated with imipramine, fluoxetine and reboxetine was isolated according to manufacturer's instructions (NucleoSpin RNA II kit, Macherey-Nagel, Germany). Total RNA was isolated separately from each individual cortex (n= 8/group).

The frozen PFC samples were lysed and homogenized in the presence of a highly denaturing ß-mercaptoethanol containing buffer, which immediately inactivates RNases. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where the total RNA bound to the membrane and contaminants were washed away. RNA was then eluted in 40 µL RNase-free water. The eluates were stored at -80°C.

Real time-PCR. RT-PCR was used to validate the differential expression of *Hdac5* and *Sirt2*. Reverse transcription to cDNA was performed using random hexamers as

primers and Superscript reverse transcriptase III (Invitrogen, Cergy Pontoise, France) with 0.3 mg total RNA for each sample. The eluates were stored at -20°C.

RT-PCR was performed in an ABI PRISM 7000 HT Sequence Detection System following manufacturer's recommendations (Applied Biosystems, CA, USA). Thermal cycling conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. Primers for mice genes *hdac5* (Mn01246076_m1) and *sirt2* (Mn01149204_m1) were used (Applied Byosistems, CA, USA). Every cDNA prepared was used in triplicate for the RT-PCR procedures for each gene tested, and the results were calculated as the average of triplicated results. PCR products were analyzed using the SDS 2.3 and the RQ Manager 1.2 Software (Applied Biosystems, CA, USA). 18S (Hs99999901_s1) was employed as an internal control to normalize RNA amount used from different samples.

Samples were analyzed by the double delta CT (ΔΔCT) method. Delta CT (ΔCT) values represent normalized target genes levels with respect to the internal control. Normalization was based on a single reference housekeeping gene (18s). Delta CT (ΔΔCT) values were calculated as the ΔCT of each test sample (imipramine, fluoxetine, reboxetine) minus the mean ΔCT of the calibrator samples (controls) for each target gene (*hdac5* and *sirt2*). The fold change was calculated using the equation $2(-\Delta\Delta CT)$.

8. Western Blot studies

SH-SY5Y cells, scrapped from 6 well cell culture clusters (Costar, Corning, NY, USA) with Dubelcco's Phosphate Buffered Saline (DPBS; gibco, Life Technologies, Paisley, UK) were centrifuged (827 g for 5 minutes). Each pellet from the cell cultures or the prefrontal cortex (PFC) dissected from the mice was 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 14,000 g at 4ºC for 20 min and the supernatant aliquoted and stored at -80ºC. Protein concentration was determined by Bradford (BIO-RAD, Hercules, CA, USA).

Western-blot. 20μg of total protein 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, they were separated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (8%) under reducing conditions and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Bioscience). The trans-blots were blocked for 1 hour with 10% skimmed milk powder in TBS buffer containing 0.1% Tween20 and then incubated overnight at 4ºC with the following primary antibodies: rabbit polyclonal anti-vesicular glutamate transporter 1 (VGLUT1; 1:2,000, kindly donated by Dr. S. El Mestikawy, Paris, France and previously validated by Herzog *et al*. 2001); rabbit polyclonal anti-brain derived neurotrophic factor (BDNF; 1:1,000, #sc-546; Santa Cruz Biotechnology, Wembley, UK); rabbit polyclonal anti-acetylated histone 3 (AcH3; 1:1,000) and rabbit polyclonal anti-acetylated histone 4 (AcH4; 1:1,000) (#06-942 and #07-329, respectively; MerckMillipore, Billerica, MA, USA); rabbit polyclonal anti-phosphorylated histone deacetylase 5 (p-HDAC5; 1:1,000, #Y011193; Applied Biological Materials, Richmond, BC, Canada); mouse monoclonal anti-histone deacetylase 5 (HDAC5; 1:1,000), rabbit monoclonal anti-sirtuin 2 (SIRT2; 1:1,000), mouse polyclonal anti-acetylated α-tubuline (1:10,000) and mouse polyclonal anti-β-actin (1:10,000) (#H4538, #S8447, #T7451 and #A1978, respectively; Sigma-Aldrich, St. Louis, MO, USA). These antibodies are commercially available and have been extensively used for their specificity.

Secondary antibodies conjugated to IRDye 800CW or IRDye 680CW (LI-COR BIOSCIENCES, Lincoln, NE, USA) were diluted up to 1:5,000 in TBS with 5% BSA. Bands were visualized using ODYSSEY Infrared Imaging System (LI-COR BIOSCIENCES, Lincoln, NE, USA). β-actin was used as loading control. Results were expressed as the percentage of optical density (O.D.) values relative to saline treated animals or control cells (DMSO) in cell culture experiments.

9. Immunofluorescence studies in SH-SY5Y cells

Human SH-SY5Y neuroblastoma cells were seeded in glass bottom culture dishes (MatTek, Ashland, MA, USA) in 24 well-plates (Greiner Bio-one, Monroe, NC, USA). Treatments were performed after 24 hours in serum-free media, and then cells were prefixed with 2% paraformaldehyde for 2 minutes and fixed with 4% paraformaldehyde (PFA, Histolab, Gothenburg, Sweden) for 20 minutes. Afterwards, cells were washed three times with PBS. All cover slips were blocked for 30 minutes in PBS with 0.1% Triton-X and 1% bovine serum albumin (all from Sigma-Aldrich, St. Louis, MO, USA). The primary antibody used was mouse monoclonal anti histone deacetylase 5 1:100 (HDAC5; #H4538; Sigma Aldrich, St. Louis, MO, USA). The secondary antibody used was Alexa fluor 488 donkey anti-mouse (1:1,000). Cover slips were first incubated overnight with the primary antibodies at 4ºC in humid chamber and then for 30 minutes at room temperature with secondary antibodies and DAPI (Invitrogen, Eugene, Oregon, USA) to identify the nuclei of cell bodies. Finally, the cover slips were rinsed in PBS and mounted using Immu-mount (Thermo Scientific, Kalamazoo, MI, USA). The cover slips were thoroughly washed in PBS between different steps. The primary antibody was omitted as a negative control. Confocal imaging was performed with a Zeiss LSM 510 META confocal laser scanning system (Carl Zeiss, Jena, Germany). The fluorescence of DAPI and Alexa 488 were recorded through separate channels with a 40× lens. Images were obtained using the Zen software (Zeiss).

10. TaqMan Low Density Arrays (TLDAs)

RNA extraction and RT-PCR. In order to study the expression of the HDAC superfamily enzymes genes in the prefrontal cortex of VGLUT1+/- and WT littermate mice TaqMan Low Density Arrays (TLDA) microfluidic card technology from Applied Biosystems (Foster City, CA, USA) was used. Total RNA was isolated separately from each individual frozen prefrontal cortex sample as describe above. As previously described by Tordera *et al*. 2011, for each tissue sample, 500 ng of reverse-transcribed RNA were diluted to 50 µL with sterile water, combined with an equal volume of TaqMan Universal PCR Master Mix (2x; Applied Biosystems, Foster City, CA, USA), mixed

by inversion, and spun briefly in an Eppendorf® 5415C microcentrifuge (Brinkmann Instruments, Westbury, NY, USA). After TLDAs were brought to room temperature, 100 µL master mix were loaded into each port connected to reaction wells. TLDAs were placed in Sorvall®/Heraeus® Custom Buckets (Applied Biosystems, Foster City, CA, USA) and centrifuged in a Sorvall Legend™ centrifuge (Kendro Scientific, Asheville, NC, USA) for 1 min at 331 for g followed closely by a second 1-min centrifugation at 331 for g. Cards with excess sample in the fill reservoir were spun for an additional 1 min. Immediately following centrifugation, the cards were sealed with a TaqMan LDA Stylus Staker (Applied Biosystems, Foster City, CA, USA), and the loading ports excised. Quantitative RT-PCR amplifications were run on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a TaqMan LDA cycling block and an automation accessory upgrade. Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each test sample was processed in duplicate on individual TLDA cards, thus allowing eight samples to be processed on each card.

Analysis of quantitative RT-PCR data. RT-PCR TaqMan instrumentation monitors gene-specific products with fluorescent dye chemistry. A cycle threshold (CT) for each reaction is the number of cycles at which the reaction crosses a selected threshold. The threshold is defined as a straight line drawn above noise/ baseline and positioned within the linear region of the semi-log amplification plot. The fewer cycles required to reach threshold fluorescence intensity, the lower the CT value and the greater the initial amount of input target. Results for each target on TLDAs were quantified concurrently using the same baseline and threshold for a target gene in order to limit interplate errors in the analysis.

Samples were analyzed by a double delta CT (ΔΔCT) method. Delta CT (ΔCT) values represent normalized target genes levels with respect the internal control. Normalization was based on a single reference housekeeping gene (18s). Delta CT (ΔΔCT) values were calculated as the ΔCT of each test sample (WT and VGLUT1+/- genotype) minus the mean ΔCT of the control samples for each target gene. The fold change was calculated using the equation $2(-\Delta\Delta CT)$.

11. *In vitro* affinity assays

Chemicals. For the *in vitro* assays, fluoxetine (Interchim, Mountluçon, France), reboxetine (kindly donated by Servier Laboratories, France), clorgyline and selegiline (Sigma-Aldrich, USA) were dissolved in distilled water. 33i was dissolved in DMSO, stored as frozen aliquots and diluted (1%) before each *in vitro* experiment. The radiolabelled compounds used were [^{3}H]paroxetine and [^{3}H]noradrenaline (Amersham, UK).

Affinity for SERT transporter. The compound 33i was studied for its ability to inhibit [^{3}H]paroxetine binding to the 5-HT transporter in rat PFC homogenates (Marcusson and Eriksson, 1988). Fluoxetine was used as reference compound. Briefly, rat cortical tissue was homogenized in Tris-HCl buffer (50 mM, pH 7.4) and centrifuged at 4°C for 10 min at 50,000 g. The supernatant was discharged, and the pellet was suspended in Tris buffer and preincubated at 37°C for 10 min. The suspension was then washed twice by centrifugation and resuspended up to a concentration cortical membrane (4 mg tissue/mL). Each tube was filled with 1200 µl of Tris buffer pH 7.4, containing NaCl 120 mM and KCl 5 mM, 400 µl of tissue preparation, and 400 µl of 0.1 nM [3H]paroxetine in Tris buffer and incubated by 60 min at 23°C. The final incubation volume was 2 mL, and nonspecific binding was determined using 10 µM fluoxetine, included in the Tris buffer, as the cold displacer.

Noradrenaline synaptosomal uptake. The ability of the compound 33i to inhibit [^{3}H]noradrenaline uptake in PFC synaptosomal preparations (Tordera *et al.* 2002) was studied. Reboxetine was used as reference compound. Briefly, rats were killed and the cerebral cortex was dissected and homogenized in 15 volumes of 0.32 M ice-cold sucrose. The homogenates were centrifuged for 10 min at 1,000 g and 4ºC and the supernatants centrifuged again at 48,000 g for 15 min. The resulting pellet was gently suspended in 0.27 M ice-cold sucrose. In this crude synaptosomal preparation, the protein concentration was 0.3 - 0.5 mg/mL. An aliquot (250 µL) of the synaptosomal preparation was added into 750 µL of Krebs buffer (pH 7.4) containing (mM): NaCl 115, KCl 4.97, CaCl2 1, MgSO4 1.22, KH2PO4 1.2, NaHCO3 25, glucose 11.1, pargyline 0.01,

ascorbic 1.7 and [3H]noradrenaline in the absence (total uptake) or presence of 33i (three concentrations from 10^{-5} to 10^{-9} µM). After 6 min at 37ºC, the reaction was finished by quick filtration through Whatman GF/B filters.

12. Monoaminooxidase activity assay

The effect of 33i (0.01 µM to 100 µM) on monoaminooxidase activity (total MAO, MAO-A and MAO-B activities) was studied following manufacturer's instructions (Catalog #K795-100Bio Vision, Milpitas, CA, USA). The monoaminooxidase inhibitors clorgyline and selegiline (10 µM) were used as reference compounds.

13. Adeno-associated viral (AVV) vector generation

New adeno-associated viral (AAV) vectors were cloned using the Gateway Multisite technology (Invitrogen, Carlsbad, CA). Gateway MultiSite recombination cloning is a two-step process. In the first step, DNA fragments containing flanking attB sites are cloned via the BP reaction into attPflanked pDONR vectors. The two entry clones (pENTR) created are combined with a ''destination'' vector (pDEST) via the LR reaction (a recombination reaction between attL and attR sites). The second step of MultiSite technology involves integrating the fragments contained in the entry clones into a destination vector via the LR reaction. In the LR reaction, distinct attL and attR sites ensure position and orientation-specific insertion. Finally, combined expression clones were introduced in the virus through a service facility (Molecular Imaging Research Center, MIRCen/ CEA, 18 route du Panorama, F-92265 Fontenay-aux-Roses, France).

Firstly, amplification of the VGLUT1^{mCherryminisog} chimaera for the BP reaction was performed by PCR (annealing 60°C and elongation 2 min). The mCherryminisog was a kind gift from Pr Roger Tsien. The primers used for these genes were GGG-GAC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTA-GAT-TTA-GCC-GTC-CAG-CTG-CAC and GGG-GAC-AAC-TTT-GTA-TAC-AAA-AGT-TGT-AAT-GGA-GTT-CCG-GCA-GGA-GGA-G, respectively. The B5-VGLUT1-mCherryminisog-B2 (2733bp) amplified fragment was recombined with

pDONR-P5-P2 in the BP reaction to create pENTR-L5-VGLUT1-mCherry-minisog-L2 (first entry clone). The LR recombination reaction was performed combining pENTR-L5-VGLUT1-mCherry-minisog-L2 and the second entry clone pENTR-L1-pSyn-R5 containing the Synapsin1 promoter sequence with pDest-AAV-R1-R2-SMD2-Rfa-WPRE. With this recombination step we created pAAV-pSyn-VGLUT1-mCherry-minisog-SMD2-Rfa-WPRE. Afterwards, the AAV vector pAAV-pSyn-VGLUT1^{mCherryminisog} was produced by the team of Dr Alexis Bemelmans together with pAAV-pSyn-YFP (Molecular Imaging Research Center, MIRCen/ CEA, 18 route du Panorama, F-92265 Fontenay-aux-Roses, France).

A schematic diagram outlining the steps of this cloning procedure and restriction map of the final product is shown in Figures 7A and 7B respectively.

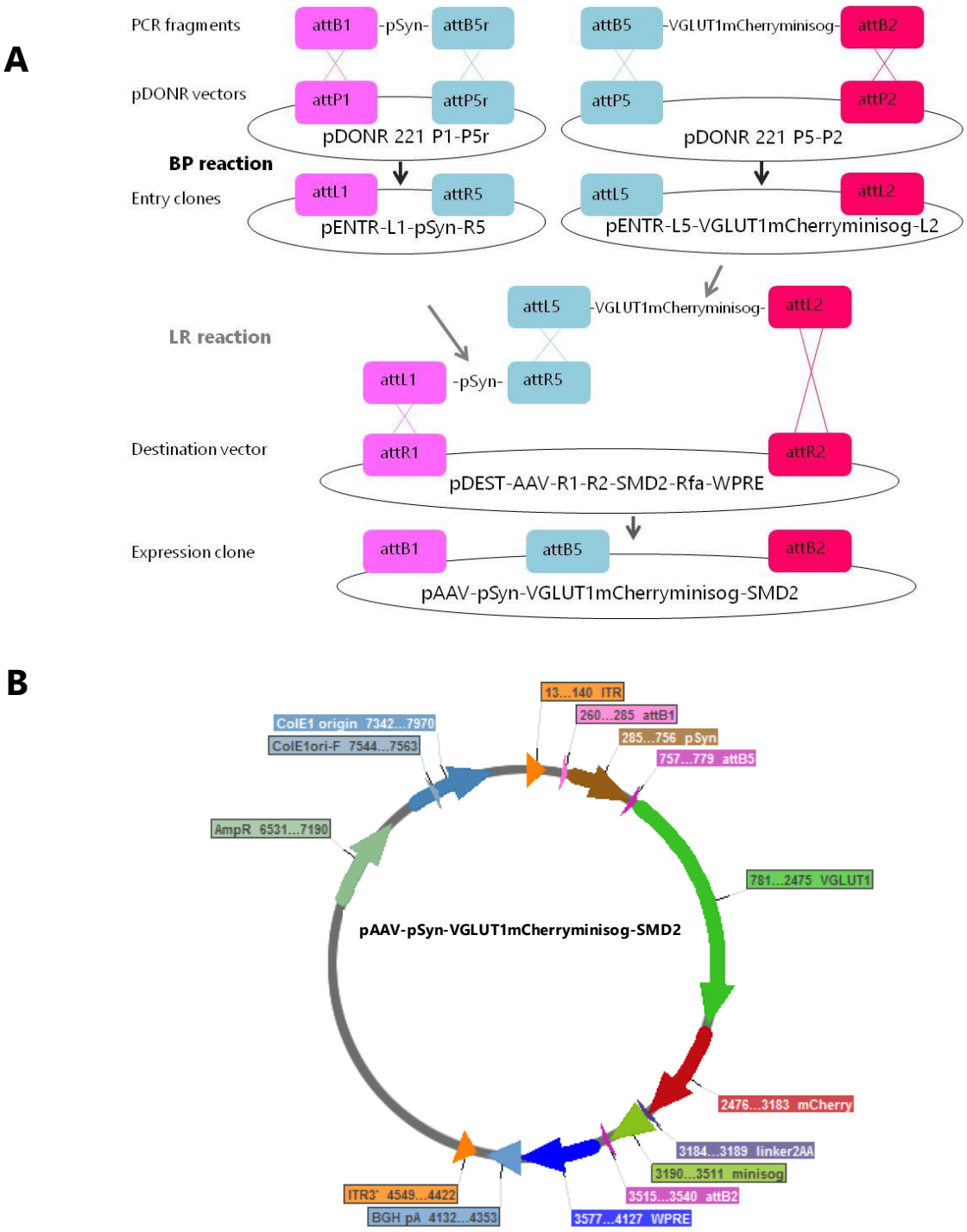


Figure 7. AAV generation. (A) Schematic diagram of Gateway MultiSite recombination cloning of the fragments VGLUT1^{mCherryminisog} and Synapsin promoter (pSyn). (B) Restriction map of the final LR reaction expression clone pAAV-pSyn-VGLUT1-mCherryminisog-SMD2 (Image obtained by Serial Cloner V2.5).

14. Expression of pAAV-pSyn-VGLUT1mCherryminisog-SMD2 in primary cultures

Plasmids were first tested in primary rat hippocampal cultures to ensure that cloning of the synapsin promoter allowed transcription of the downstream VGLUT1^{mCherryminisog} construct.

The expression clone pAAV-pSyn-VGLUT1mCherryminisog-SMD2 was tested *in vitro* in primary cultures of rat hippocampal neurons (embryo at E18 days) by electroporation. In rodents, VGLUT1 mRNA is expressed from postnatal day 14 (PN14) (Wojcik *et al.* 2004).

Briefly, cells were transiently transfected using the commercially available Amaxa MeP1 Nucleofector kit, including nucleofector solution for rat neurons and certified cuvettes, according to the manufacturer's instructions (Amaxa biosystems, Cologne, Germany). In brief, 2 µg of DNA (vector) were dissolved in 8 µL nucleofector solution for rat neurons at 4°C. 500,000 cells were suspended in 3 mL nucleofector solution for rat neurons at room temperature. After centrifugation (5 min 1,000 g), the cell suspension was mixed with the 8 µL DNA-containing solution by pipetting three times up and down. The mixture was transferred to a cuvette that was inserted into the Nucleofector device (Amaxa biosystems). After electroporation the neurons were transferred from the cuvette into dishes with 4 µL of pre-warmed DMEM 1X HorseSerum prewarmed (37°C).

This step was carried out between days 2 and 5 of the *in vitro* cultures. Four dilutions of the original batch were tested: 1/1,000; 1/10,000; 1/50,000; 1/100,000. Every 5 days the medium was changed. Images were taken between days 17 and 21.

15. Stereotaxic injections of pAAV-pSyn-VGLUT1^{mCherryminisog} and pAAV-pSyn-YFP

For stereotaxic delivery of the AAV vector, adult mice were randomly assigned to treatment with the pAAV-pSyn-VGLUT1^{mCherryminisog} or control groups injected with pAAV-pSyn-YFP (n= 9-11 mice per group) of each genotype. Mice were anesthetized by intraperitoneal 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 either pAAV-pSyn-YFP or pAAV-pSyn-VGLUT1^{mCherryminisog} (1.47 E^{12} and 2.01E^{14} viral genomes/mL, respectively) bilaterally into the IL-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/min 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 was 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.

16. Imaging of VGLUT1^{mCherryminisog} and YFP expression in the brain

Tissue preparation. The rostro-caudal extent of transgene expression of all animals used in behavioural experiments was assessed by the fluorophore YFP as control or mCherrymisinog as target.

Mice were sacrificed nine weeks post-injection (age of animals: 16 weeks). The animals received an anesthetic overdose of sodium pentobarbital (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 frontal cortex (Bregma 2.40 to 1.40) and the dorsal raphe nuclei (Bregma -4.10 to -5.40) and were stored in cryoprotectant solution at -20°C until processing.

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

Image capture. Fluorescence of YFP and VGLUT1^{mCherryminisog} was 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).

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 (Stenberg 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.

17. Sucrose intake test

Anhedonic-like behaviour was evaluated in WT and VGLUT1+/- mice by monitoring of sucrose intake (Elizalde *et al.* 2008). Mice were first trained for 1 week to drink the 2.5% sucrose solution. After this preliminary phase, mice were food deprived and exposed to the sucrose solution and tap water for 15 hours once a week. The intake baseline for the sucrose solution was established, which corresponded to the average of three consecutive measurements of sucrose intake carried out every 4-5 days.

Then, WT and VGLUT1+/- mice were divided into two subgroups matched for sucrose consumption and body weight in order to start drug treatments (imipramine or 33i, experimental design 2) or stereotaxic injections with the adeno-associated viruses (AAVs, experimental design 3). Once a week, mice were given a 15-h exposure to the sucrose solution and tap water in their home cage as described above. The position of the two bottles (right/left) was varied randomly from trial to trial. 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.

18. Behavioural tests

Spontaneous locomotor activity and open field. Locomotor activity was measured in an open field consisting of 8 black square arenas (43 x 50 x 45) using a video tracking system (Ethovision XT 11 plus multiple body point module, Noldus Information Technology, Wageningen, The Netherlands) in a softly illuminated experimental room. One mouse was placed in each cage and distance travelled (cm) was recorded during a 30 minute period. Time spent (s) in center zone (25 cm^2) was also recorded.

Social interaction test. This test measures the approach-avoidance behaviour towards an unfamiliar social target (Tsankova *et al.* 2006; Venzala *et al.* 2012). The arena is a transparent plastic open field (45 × 45 × 45 cm3) maintained in a very softly illuminated room (50 lx). Each experimental mouse was introduced into the open field and its trajectory was tracked for two consecutive sessions of 2.5 min. During the first session ("no target") the open field contained an empty metallic mesh cage (10.5 × 9 cm2) located at one end of the field. During the second session ("target"), the conditions were identical except that a social target animal (a mouse from the same strain and of similar weight) had been introduced into the cage. Between the two sessions, the experimental mouse was removed from the arena, and was placed back into its home cage for approximately 1 min. The time that the experimental mouse spent in the "interaction zone" (7 cm wide corridor surrounding the little metallic cage)

both in the "no target" and "target" conditions were recorded (Ethovision XT 11 plus multiple body point module, Noldus Information Technology, Wageningen, The Netherlands) and differences ("target-no target") were calculated for each mouse (Venzala *et al*. 2012).

Marble Burying Test. Natural burying behaviour was assessed with this test. Twelve 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 recorded.

19. Core body temperature studies: 5-HT_{1A} agonist induced hypothermia

Baseline body temperature was measured using a thermistor probe inserted 2 cm into the mice rectum seven weeks after stereotaxic injections. Mice received a subcutaneous injection of the 5-HT_{1A} receptor agonist 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) 0.4mg/kg (RBI, Wayland, MA, U.S.A) and core body temperature was measured at 30 minutes intervals for up to 90 minutes. Data were presented as the difference in core body temperature over basal values.

20. Statistical analysis

The effect of different antidepressants (imipramine, fluoxetine and reboxetine) on mRNA and protein expression of different epigenetic and synaptic plasticity markers were analyzed using One-way ANOVA (treatment) followed by post-hoc Dunnett test. The effect of the compounds 33i, MC3822, MC3823 or scopolamine on protein expression was evaluated by Student's t test.

Differences in the mRNA abundance of each HDAC enzyme as well as AAV infection efficiency were analysed using student *t*-test in the PFC of VGLUT1+/- and WT mice.

Sucrose intake test, body weight gain and differences of temperature were analyzed through two-way ANOVA (genotype by drug treatment or genotype by AAVs

treatment) with repeated measures followed by two-way ANOVA for each time point.

Different parameters measured in behavioural tests (motor activity, open field, social interaction and marble burying) were analyzed by two-way ANOVA (genotype by AAVs treatment).

All results were expressed as mean ± standard error of the mean (SEM).

RESULTS

1. Role of HDAC5 and SIRT2 in the molecular mechanisms of antidepressants

Here we have explored the role of HDAC5 and SIRT2 in the molecular mechanisms of antidepressants. Firstly, using the *in vitro* SH-SY5Y cellular model we have studied the antidepressant regulation of these epigenetic targets and their involvement in synaptic plasticity using selective HDAC's inhibitors. Specifically, the expression of the synaptic plasticity markers the brain derived neurotrophic factor (BDNF) and the vesicular glutamate transporter 1 (VGLUT1) have been studied.

1.1. Antidepressant treatment regulate HDAC5 *in vitro*

Incubation of SH-SY5Y cells with antidepressants for 2 hours revealed significant differences in HDAC5 [$F_{3,24}$= 4.01, $p<0.05$] and p-HDAC5 [$F_{3,24}$= 4.2, $p<0.05$]. Particularly, HDAC5 expression was upregulated by imipramine and reboxetine ($p<0.05$) and p-HDAC5 was significantly increased by reboxetine ($p<0.05$) (Figure 8).

Incubation of SH-SY5Y cells with antidepressants (10 μM, 24 hours) revealed changes in HDAC5 [$F_{3,\ 24}$= 6.18, $p<0.01$] and p-HDAC5 [$F_{3,24}$= 4.44, $p<0.01$] expression. Both imipramine ($p<0.05$) and reboxetine ($p<0.01$) showed increased expression of p-HDAC5. In addition, HDAC5 expression appeared to be upregulated by reboxetine ($p<0.01$) (Figure 9A).

We next studied by immunofluorescence the effect of the selective serotonin reuptake inhibitor fluoxetine and the selective noradrenaline reuptake inhibitor reboxetine (10 μM, 24 hours) on HDAC5 nucleocytoplasmic shuttling on SH-SY5Y cells. While no effect of fluoxetine treatment was observed in HDAC5 cellular localization, reboxetine treatment clearly induced nuclear export of HDAC5 to the cytoplasm (Figure 9B).

Subsequently, we looked for selective HDAC5 inhibitors to test the role of HDAC5 in the expression of synaptic plasticity markers.

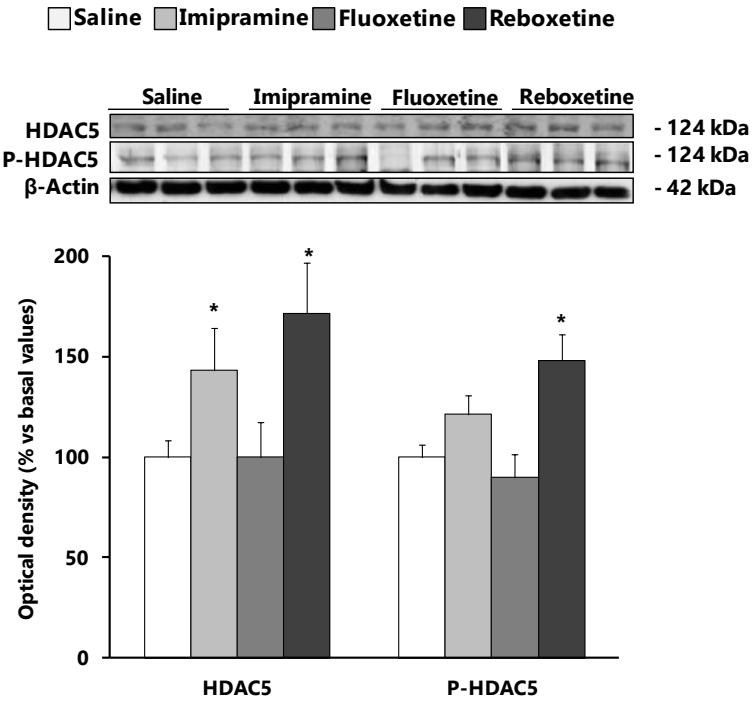


Figure 8. Effect of 2 hour incubation with antidepressants on HDAC5 and p-HDAC5 expression *in vitro*. Effect of imipramine, fluoxetine and reboxetine (10 μM, 2 hours) on HDAC5 and p-HDAC5 expression in SH-SY5Y cell cultures. Values show the mean ± SEM of relative abundance of protein expression compared to controls. *p<0.05 *vs* corresponding controls. One-way ANOVA followed by Dunnett test. Abbreviations: HDAC5, histone deacetylase 5; p-HDAC5, phosphorylated histone deacetylase 5.

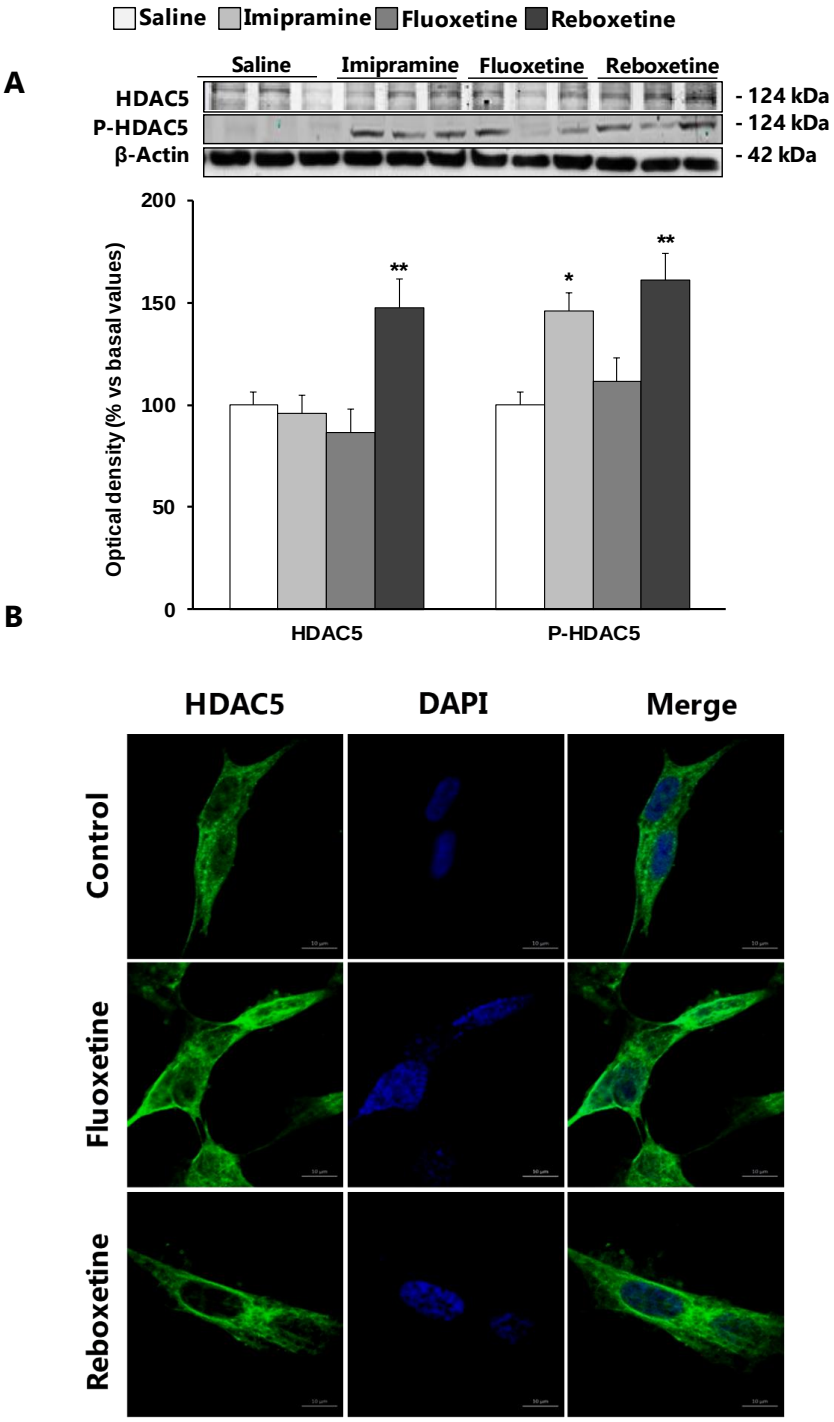
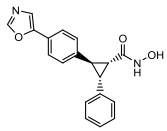
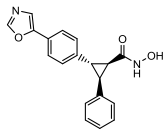


Figure 9. Antidepressant regulation of HDAC5 *in vitro*. (A) Effect of imipramine, fluoxetine and reboxetine (10 μM, 24 hours) on HDAC5 and p-HDAC5 expression in SH-SY5Y cell cultures. Data show the mean ± SEM of relative abundance of protein expression compared to controls. **p<0.01, *p<0.05 *vs* corresponding controls. One-way ANOVA followed by Dunnett test. Abbreviations: HDAC5, histone deacetylase 5; p-HDAC5, phosphorylated histone deacetylase 5. (B) Representative photomicrographs of HDAC5 and nuclear (DAPI) immunofluorescence in SH-SY5Y cell cultures incubated with vehicle (1% DMSO), fluoxetine and reboxetine (10 μM, 24 hours). HDAC5+ (green), DAPI+ (blue) and double (HDAC5+/DAPI+) cells are shown. Scale bar: 10 μm.

1.2. MC3822 and MC3823 affinity to the different HDAC enzymes

Data from Table 1 show the effect of MC3822 and MC3823 in HDAC1-9 activity. MC3822 clearly selectively inhibits HDAC4 and 5 at double digit nanomolar level (IC50= 0.038 and 0.012 respectively), HDAC7, 8 and 9 at submicromolar level (IC50= 0.13, 0.31 and 0.38 respectively), and HDAC6 at single-digit micromolar concentration. The isomer MC3823 displayed similar trend of HDAC inhibition with lower potency showing micromolar inhibition for HDAC4-9. Moreover, MC3822 was totally inactive against HDAC1-3.

Table 1. Biochemical activity of MC3822 and MC3823 against HDAC isoforms.

Cpd	HDAC isoforms (IC50, µM or % of inhibition at 200 µM)								
	HDAC1	HDAC2	HDAC3	HDAC4	HDAC5	HDAC6	HDAC7	HDAC8	HDAC9
MC3822  (1R,2R,3R)-N-hydroxy-2-(4-(oxazol-5-yl)phenyl)-3-phenylcyclopropane-1-carboxamide	644	23%	19%	0.038	0.012	3.61	0.13	0.31	0.38
MC3823  (1S,2S,3S)-N-hydroxy-2-(4-(oxazol-5-yl)phenyl)-3-phenylcyclopropane-1-carboxamide	42%	20%	0%	7.28	1.54	11.4	18.2	2.25	26.6

1.3. HDAC5 inhibition enhances histone acetylation and synaptic plasticity markers

We next studied the *in vitro* effect of the selective HDAC5 inhibitors MC3822 and MC3823 on histone acetylation and synaptic plasticity markers. Incubation of SH-SY5Y cell line with these compounds (5 μM, 24 hours) regulated both AcH3 [$F_{2,14}$= 3.24, $p<0.05$] and AcH4 [$F_{2,14}$= 2.75] levels. Between the two enantiomers the one with higher potency to inhibit HDAC5, MC3822, increased significantly AcH3 and AcH4 expression ($p<0.05$) (Figure 10A).

Subsequently, we tested the effect of MC3822 on synaptic plasticity markers expression levels. MC3822 (5 μM, 24 hours) induced a significant upregulation of VGLUT1 ($p<0.05$) and BDNF expression levels ($p<0.01$) (Figure 10B).

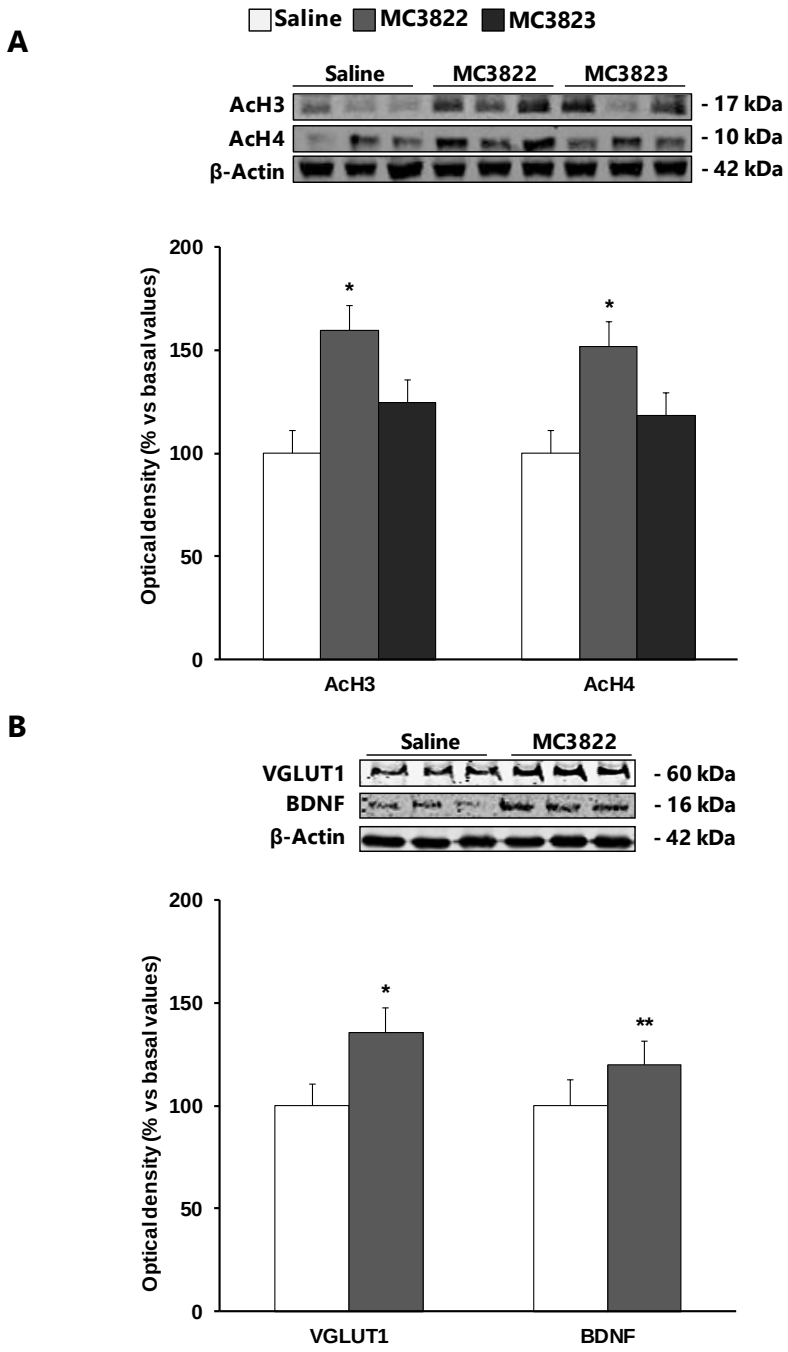


Figure 10. HDAC5 inhibition increases histone acetylation and synaptic plasticity markers *in vitro*. Effect of MC3822 and MC3823 (5 μM, 24 hours) on (A) AcH3 and AcH4 expression and on (B) VGLUT1 and BDNF expression SH-SY5Y cell cultures. Values show the mean ± SEM of relative abundance of protein expression compared to controls. **p<0.01, *p<0.05 *vs* corresponding controls. One-way ANOVA followed by Dunnett test in (A) and Student t-test in (B). Abbreviations: AcH3, acetylated histone 3; AcH4, acetylated histone 4; VGLUT1, vesicular glutamate transporter 1; BDNF, brain-derived neurotrophic factor.

1.4. Antidepressant regulation of SIRT2 *in vitro*

In addition to HDAC5, the antidepressant regulation of SIRT2 was studied *in vitro*. Incubation of SH-SY5Y cells with antidepressants (10 μM, 2 hours) showed that only fluoxetine treatment significantly downregulated SIRT2 [$F_{3,24}$= 3.51, $p<0.05$] and increased acetylated α-tubulin [$F_{3,24}$= 4.21, $p<0.05$] ($p<0.01$) (Figure 11). However, 24 hour incubation revealed changes in SIRT2 [$F_{3,24}$= 8.23, $p<0.01$] and acetylated α-tubulin [$F_{3,24}$= 3.12, $p<0.05$]. SIRT2 was clearly downregulated by the three antidepressants ($p<0.001$ imipramine and $p<0.01$ for fluoxetine and reboxetine) and increased acetylated α-tubulin was observed following imipramine and fluoxetine ($p<0.05$) (Figure 12).

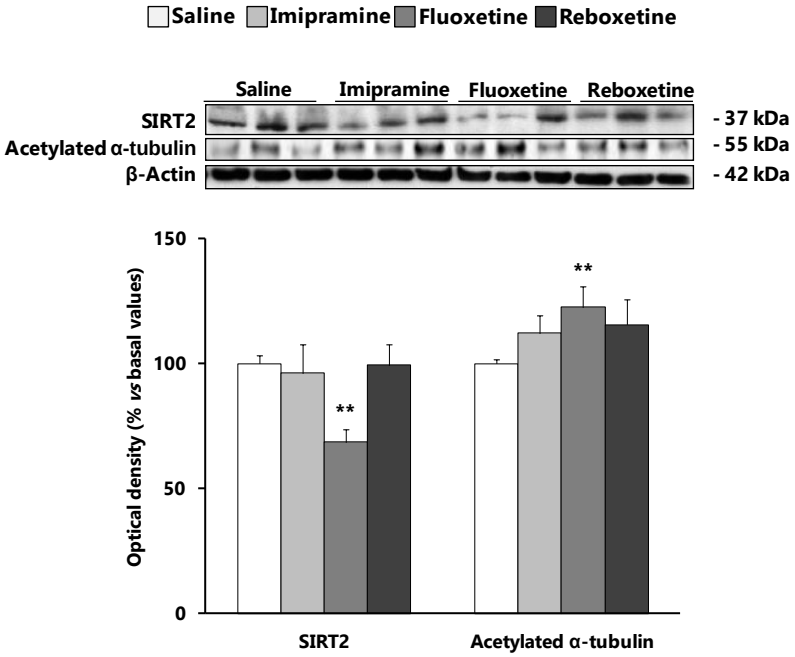


Figure 11. Effect of 2 hour incubation with antidepressants on SIRT2 expression *in vitro*. Effect of imipramine, fluoxetine and reboxetine (10 μM, 2 hours) on SIRT2 and acetylated α-tubulin expression in SH-SY5Y cell cultures. Values show the mean ± SEM of relative abundance of protein expression compared to controls. **p<0.01 *vs* corresponding controls. One-way ANOVA followed by Dunnett test. Abbreviation: SIRT2, sirtuin 2.

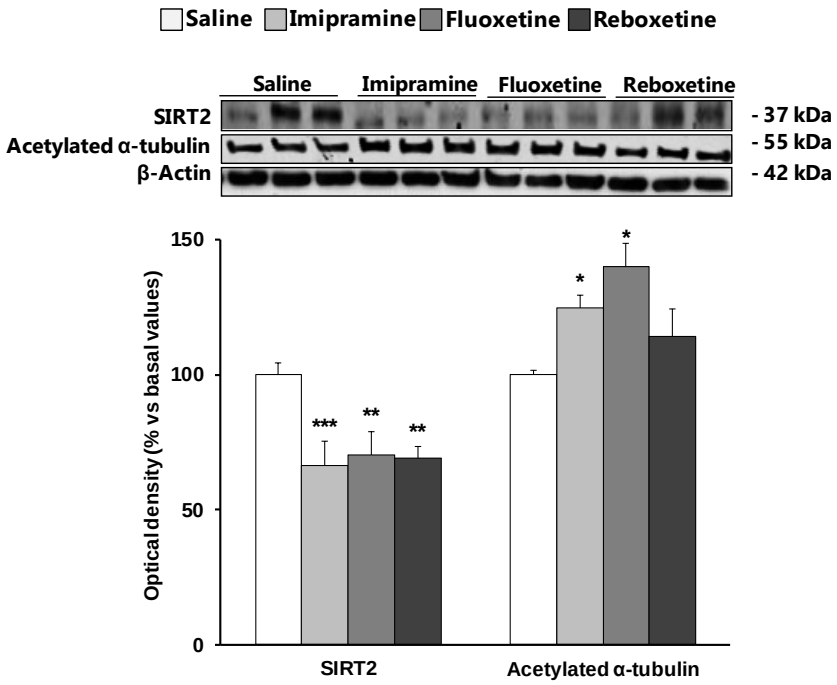


Figure 12. Antidepressant regulation of SIRT2 *in vitro*. Effect of imipramine, fluoxetine and reboxetine (10 µM, 24 hours) on SIRT2 and acetylated α-tubulin expression in SH-SY5Y cell cultures. Data show the mean ± SEM of relative abundance of mRNA or protein expression compared to controls. ***p<0.001, **p<0.01, *p<0.05 *vs* corresponding controls. One-way ANOVA followed by Dunnett test. Abbreviations: SIRT2, Sirtuin 2.

1.5. SIRT2 inhibition enhances histone acetylation and synaptic plasticity markers

Subsequently, the *in vitro* effect of the SIRT2 inhibitor 33i (Suzuki *et al*. 2012) (5 µM, 24 hours) on histone acetylation and synaptic plasticity markers was studied here. A significant upregulation of both AcH3 and AcH4 expression was observed ($p<0.01$) (Figure 13A). In addition, the synaptic plasticity markers VGLUT1 and BDNF were also upregulated by 33i ($p<0.05$) (Figure 13B).

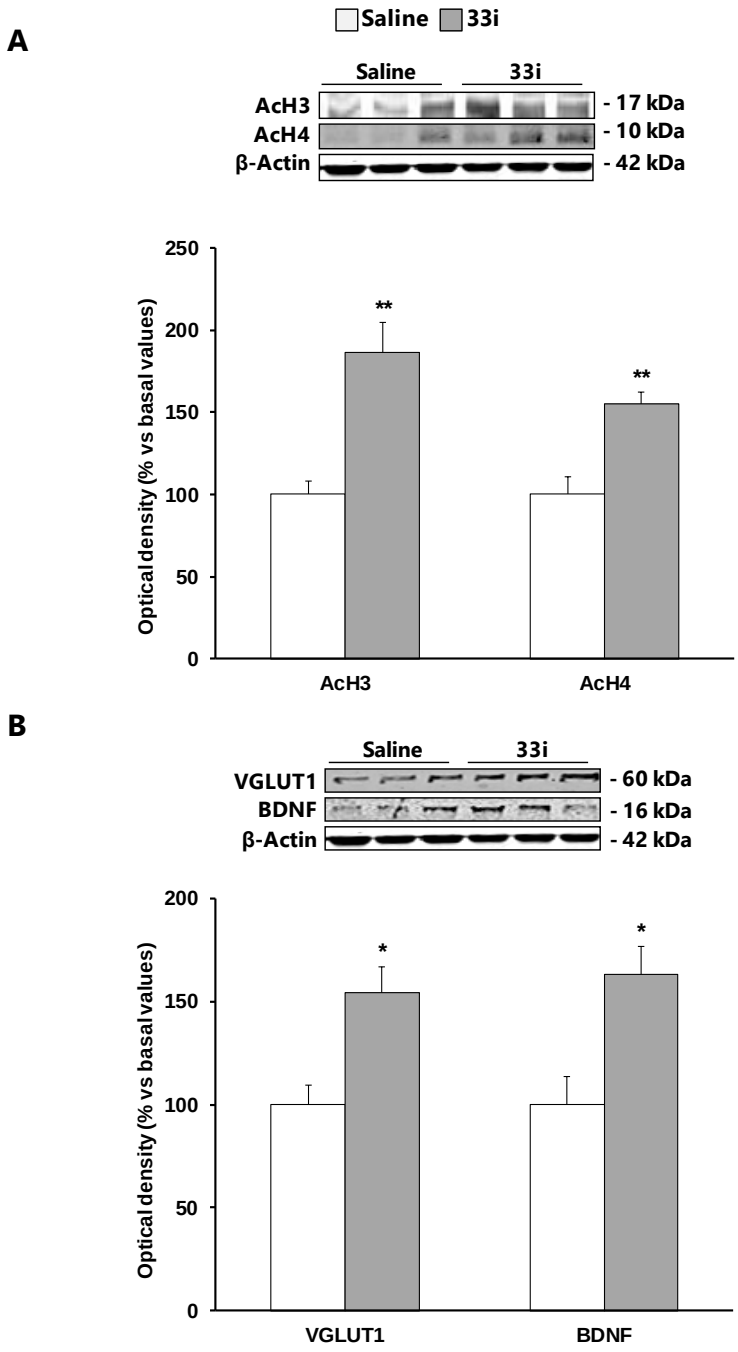


Figure 13. SIRT2 inhibition increases histone acetylation and synaptic plasticity markers *in vitro*. Effect of 33i (5 μM, 24 hours) on (A) AcH3 and AcH4 expression and on (B) VGLUT1 and BDNF expression SH-SY5Y cell cultures. Values show the mean ± SEM of relative abundance of protein expression compared to controls. **p<0.01, *p<0.05 *vs* corresponding controls, Student t-test.

1.6. Effect of MC3822 and 33i on HDAC5 and SIRT2 expression *in vitro*

We next studied whether the SIRT2 inhibitor 33i and the HDAC5 inhibitor MC3822 affect to the expression of SIRT2 and acetylated α-tubulin together with HDAC5 and p-HDAC5. Particularly, 33i (5 μM, 24 hours) increased acetylated α-tubulin ($p<0.05$) and p-HDAC5 ($p<0.001$) in SH-SY5Y cells (Figures 14A and 14B). On the other hand, neither of these proteins were affected by MC3822 (Figures 14C and 14D).

Subsequently the *in vitro* effect of the selective SIRT2 inhibitor 33i (5 μM, 24 hours) on HDAC5 localization was studied in SH-SY5Y cells. We observed that 33i clearly induced nuclear export of HDAC5 to the cytoplasm (Figure 14E).

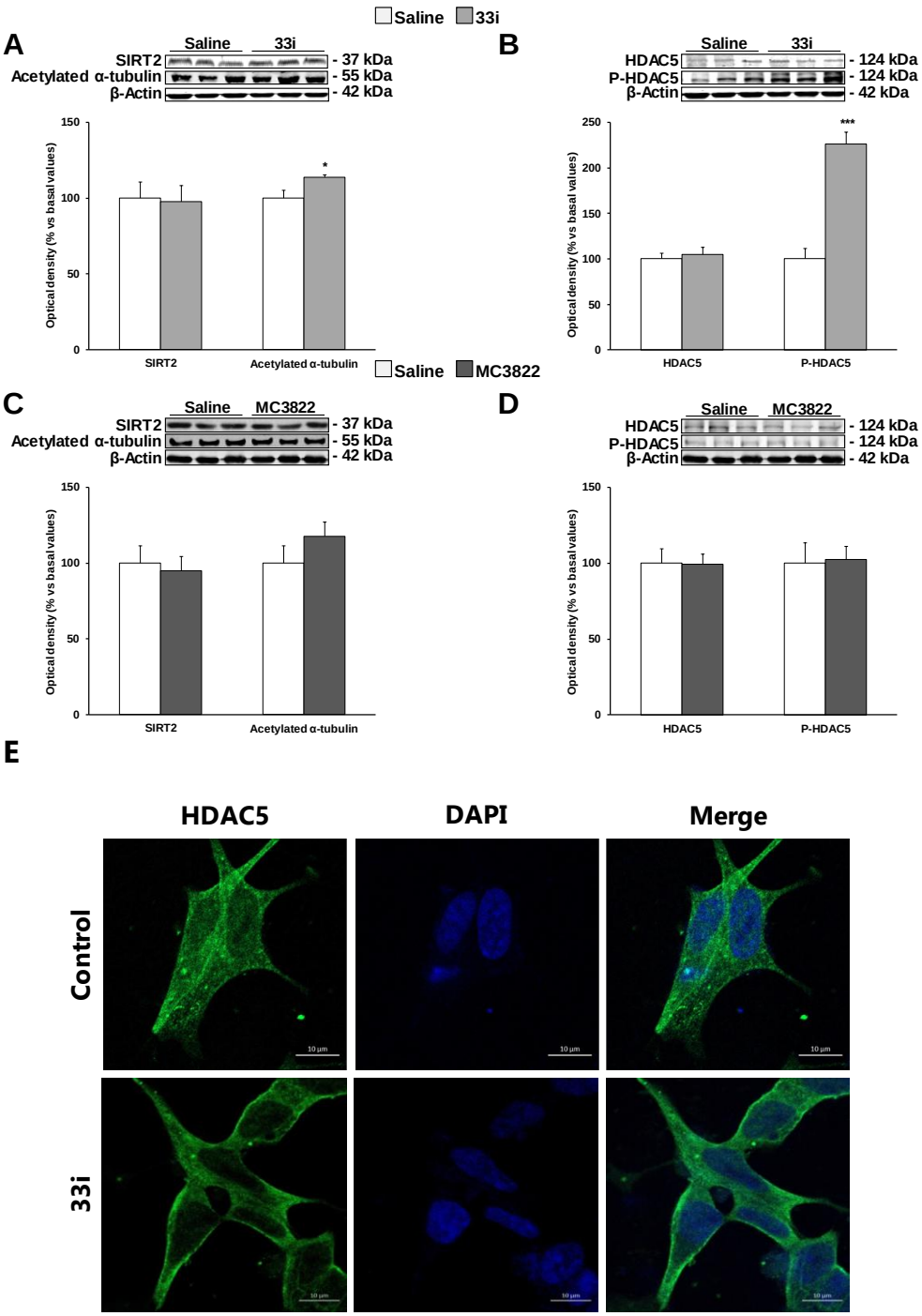


Figure 14. SIRT2 inhibition regulates HDAC5 cellular localization in SH-SY5Y cell cultures. (A, B) Effect of the HDAC5 inhibitor MC3822 on (A) SIRT2 and acetylated α-tubulin and (B) HDAC5 and p-HDAC5 expression in SH-SY5Y cell cultures. (C-E). Effect of the SIRT2 inhibitor 33i (5 µM, 24 hours) on (C) SIRT2 and acetylated α-tubulin, (D) HDAC5 and p-HDAC5 expression and on (E) HDAC5 cellular localization in SH-SY5Y cell cultures. Values show the mean ± SEM of relative abundance of protein expression compared to controls. ***p<0.01, *p<0.05 *vs* corresponding controls, Student t-test. In (E), representative photomicrographs of HDAC5 and nuclear (DAPI) immunofluorescence in SH-SY5Y cell cultures incubated with vehicle (1% DMSO) or 33i (5 µM, 24 hours). HDAC5+ (green), DAPI+ (blue) and double (HDAC5+/DAPI+) cells are shown. Scale bar: 10 µm.

1.7. Antidepressant treatment regulate histone acetylation and synaptic plasticity markers *in vitro*

In order to recapitulate previous *in vivo* findings (Erburu *et al*. 2015a), we studied the antidepressant regulation of histone acetylation and synaptic plasticity markers *in vitro.*

Incubation of SH-SY5Y cells with imipramine, fluoxetine and reboxetine (10 μM, 2 hours) influenced AcH3 [$F_{3,24}$= 4.87, p<0.01] and AcH4 [$F_{3,24}$= 6.35, p<0.001] expression levels. Specifically, AcH3 expression was significantly increased by imipramine (p<0.05) and fluoxetine (p<0.01). Similarly, AcH4 was increased by fluoxetine (p<0.001) and imipramine (p<0.05) (Figure 15A). However, incubation of SH-SY5Y cells with antidepressants for 2 hours did not affect to the expression of VGLUT1 and BDNF (Figure 15B).

A pronounced increase on AcH3 [$F_{3,24}$= 8.15, p<0.001] and AcH4 [$F_{3,24}$= 6.01, p<0.01] could be observed on SH-SY5Y cells, following 24 hour antidepressant treatment (10 μM). Both AcH3 and AcH4 expression were increased by imipramine (p<0.01), fluoxetine (p<0.001 for AcH3 and p<0.01 for AcH4) and reboxetine (p<0.05) (Figure 16A).

Incubation of SH-SY5Y cells with imipramine and fluoxetine (10 μM, 24 hours) also regulated the expression of these synaptic plasticity markers [$F_{3,24}$= 16.99, p<0.001 and $F_{3,24}$= 4.06, p<0.001 for VGLUT1 and BDNF respectively]. Both imipramine and fluoxetine increased VGLUT1 (p<0.01; p<0.001) and BDNF expression levels (p<0.01; p<0.05; for VGLUT1 and BDNF, respectively) (Figure 16B). Although no statistically significant, reboxetine showed a tendency to increase VGLUT1 and BDNF compared to saline.

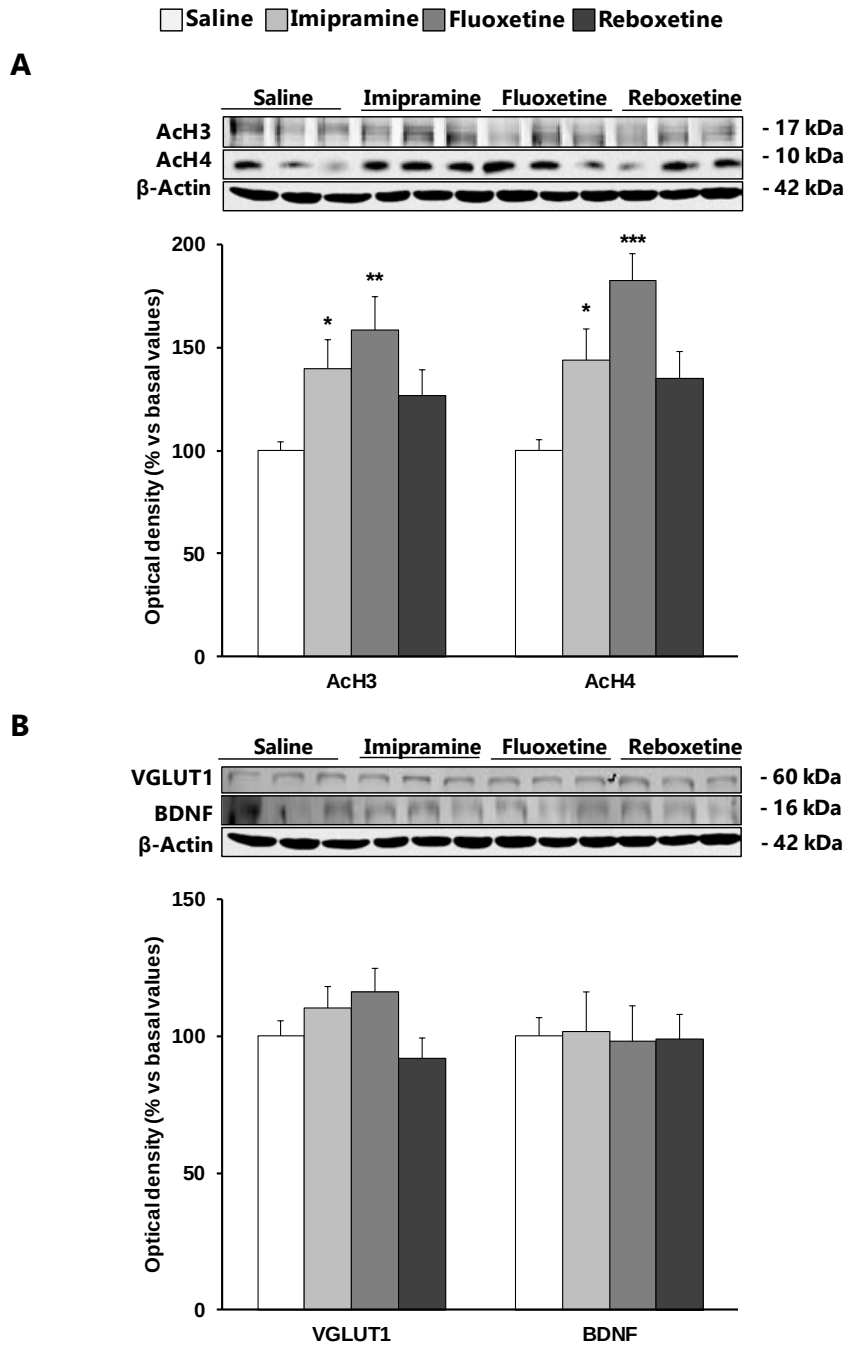


Figure 15. Effect of 2 hour incubation with antidepressants on histone acetylation, VGLUT1 and BDNF *in vitro*. (A) Effect of imipramine, fluoxetine and reboxetine (10 μM, 2 hours) on AcH3 and AcH4 expression and (B) on VGLUT1 and BDNF expression in SH-SY5Y cell cultures. Values show the mean ± SEM of relative abundance of protein expression compared to controls. ***p<0.001, **p<0.01, *p<0.05 *vs* corresponding controls. One-way ANOVA followed by Dunnett test. Abbreviations: AcH3, acetylated histone 3; AcH4, acetylated histone 4; VGLUT1, vesicular glutamate transporter 1; BDNF, brain-derived neurotrophic factor.

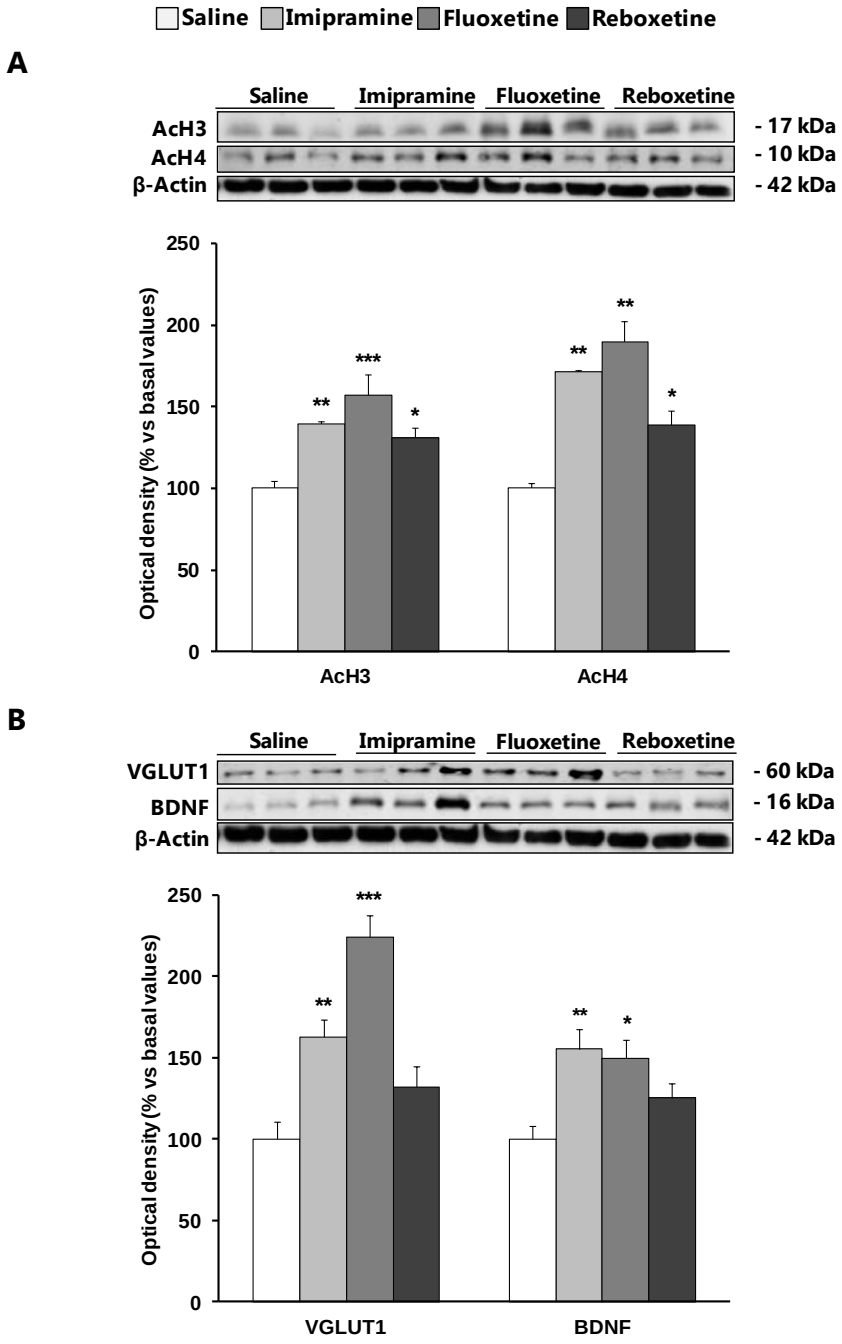


Figure 16. Antidepressant incubation for 24 hours increases histone acetylation, VGLUT1 and BDNF *in vitro*. (A) Effect of imipramine, fluoxetine and reboxetine (10 μM, 24 hours) on AcH3 and AcH4 expression and (B) on VGLUT1 and BDNF expression SH-SY5Y cell cultures. Values show the mean ± SEM of relative abundance of protein expression compared to controls. ***p<0.001, **p<0.01, *p<0.05 *vs* corresponding controls. One-way ANOVA followed by Dunnett test. Abbreviations: AcH3, acetylated histone 3; AcH4, acetylated histone 4; VGLUT1, vesicular glutamate transporter 1; BDNF, brain-derived neurotrophic factor.

1.8. Muscarinic receptor blockade does not affect histone acetylation and synaptic plasticity markers *in vitro*

In order to discard any possible anticholinergic action on epigenetic targets, the antimuscarinic drug scopolamine was studied. Incubation with the antimuscarinic drug scopolamine (10 μM, 24 hours), did not affect the expression of AcH3, AcH4 (Figure 17A) as well as VGLUT1 and BDNF (Figure 17B).

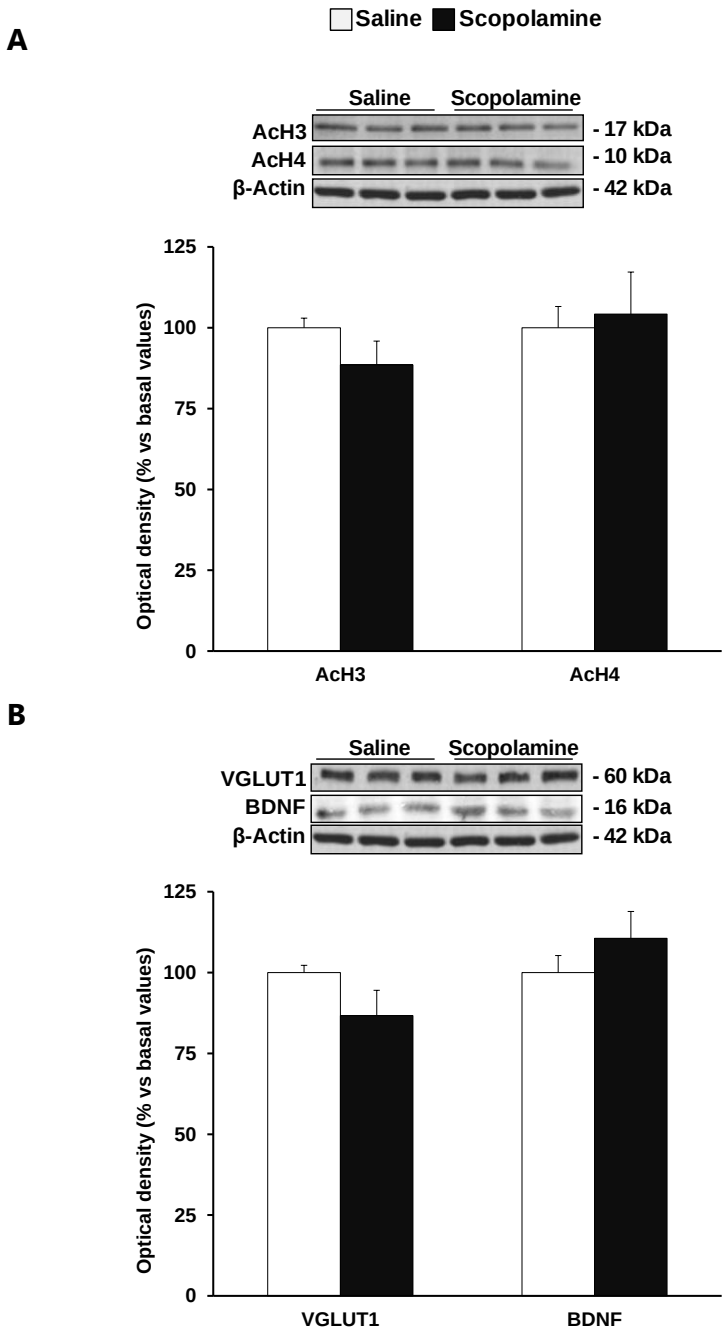


Figure 17. Scopolamine does not affect histone acetylation or synaptic plasticity markers *in vitro*. Effect of scopolamine (10 μM, 24 hours) on (A) AcH3 and AcH4 expression and on (B) VGLUT1 and BDNF expression SH-SY5Y cell cultures. Values show the mean ± SEM of relative abundance of protein expression compared to controls. Student t-test.

1.9. Chronic antidepressant treatment regulate HDAC5 and SIRT2 *in vivo*

Finally, the long term antidepressant regulation of HDAC5 and SIRT2 was studied in the mice PFC.

RT-PCR studies showed that Hdac5 mRNA abundance in PFC was affected by chronic antidepressant treatment [$F_{3,24}$= 6.08, $p<0.01$]. Both repeated imipramine ($p<0.05$) and reboxetine ($p<0.01$) significantly increased the expression of this gene compared to control saline treated mice (Figure 18A). On the other hand, while HDAC5 protein expression in the PFC was not affected by chronic antidepressants [$F_{3,24}$= 2.15, $p>0.05$], changes in the expression of the phosphorylated form (p-HDAC5) were observed [$F_{3,24}$= 4.29, $p<0.001$]. Repeated imipramine and reboxetine treatment confirmed a significant p-HDAC5 upregulation ($p<0.05$) (Figure 18B).

Sirt2 mRNA expression in PFC was influenced by chronic antidepressant treatment [$F_{3,24}$= 5.47, $p<0.05$]. Chronic fluoxetine treatment significantly downregulated Sirt2 mRNA levels ($p<0.05$) compared to control saline (Figure 18C). Moreover, significant effects of chronic antidepressant treatment on SIRT2 [$F_{3,24}$= 5.28, $p<0.01$] and SIRT2 cytoplasmic substrate acetylated α-tubulin [$F_{3,24}$= 4.68, $p<0.01$] expression levels were observed. Specifically, SIRT2 was downregulated by the three antidepressants compared to saline treated mice ($p<0.05$ for imipramine and fluoxetine; $p<0.01$ for reboxetine). Moreover, imipramine and reboxetine increased acetylated α-tubulin ($p<0.05$) (Figure 18D).

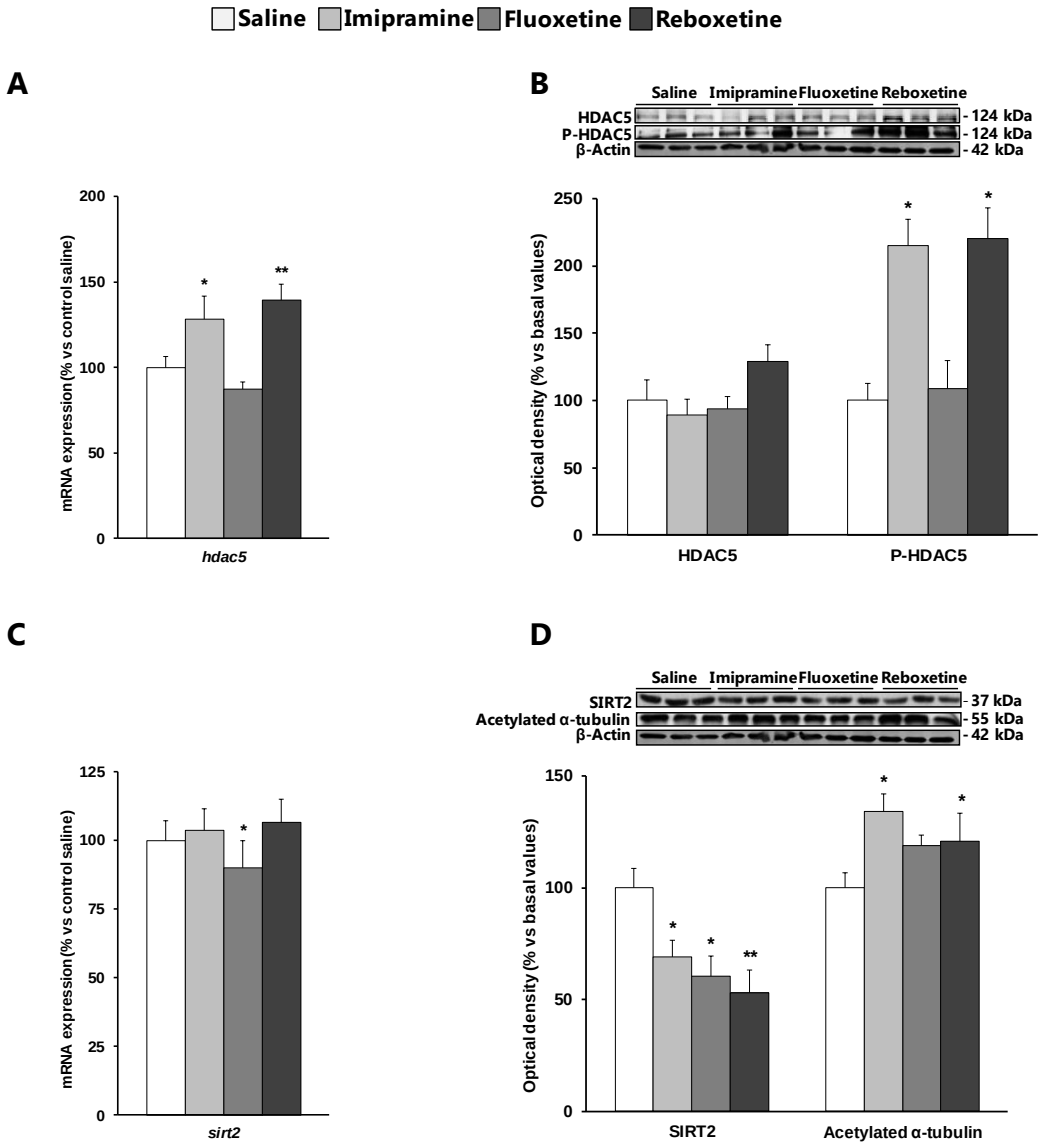


Figure 18. Antidepressant regulation of HDAC5 and SIRT2 *in vivo*. (A, B) Effect of chronic treatment with imipramine (10 mg/kg), fluoxetine (15 mg/kg) and reboxetine (15 mg/kg) for three weeks on (A) Hdac5 mRNA abundance and (B) HDAC5 and p-HDAC5 expression in mice prefrontal cortex. (C, D) and on (C) Sirt2 mRNA abundance and (D) SIRT2 and cytoplasmic SIRT2 substrate acetylated α-tubulin in mice prefrontal cortex. Data show the mean ± SEM of relative abundance of mRNA or protein expression compared to controls. **p<0.01, *p<0.05 *versus* corresponding controls. One-way ANOVA followed by Dunnett test. Abbreviations: HDAC5, histone deacetylase 5; p-HDAC5, phosphorylated histone deacetylase 5; SIRT2, Sirtuin 2.

1.10. Chronic antidepressant treatment regulate epigenetic and synaptic plasticity markers in the mouse PFC

The long-term antidepressant regulation of all these epigenetic and synaptic plasticity markers in the mice PFC was studied comparatively to the *in vitro* studies. Chronic antidepressant treatment for 21 days regulated the expression of AcH3 and AcH4. Specifically, the three antidepressants caused an increase ($p<0.05$) on AcH3 expression [$F_{3,24}= 3.71$, $p<0.05$]. In addition, AcH4 levels were increased by imipramine ($p<0.05$), reboxetine ($p<0.01$) and fluoxetine ($p<0.001$) [$F_{3,24}= 9.83$, $p<0.001$] (Figure 19A).

In addition, chronic antidepressant treatment for 21 days regulated the expression of the synaptic vesicle protein VGLUT1 [$F_{3,24}= 4.23$, $p<0.01$] and the neurotrophin BDNF [$F_{3,24}= 3.00$, $p<0.05$] in the prefrontal cortex (PFC). In particular, VGLUT1 expression was significantly increased by imipramine (10 mg/kg) ($p<0.05$), fluoxetine (15 mg/kg) ($p<0.01$) and reboxetine (15 mg/kg) ($p<0.05$) following three weeks of treatment. Moreover, the expression of BDNF was significantly increased after imipramine, fluoxetine and reboxetine ($p<0.05$) (Figure 19B).

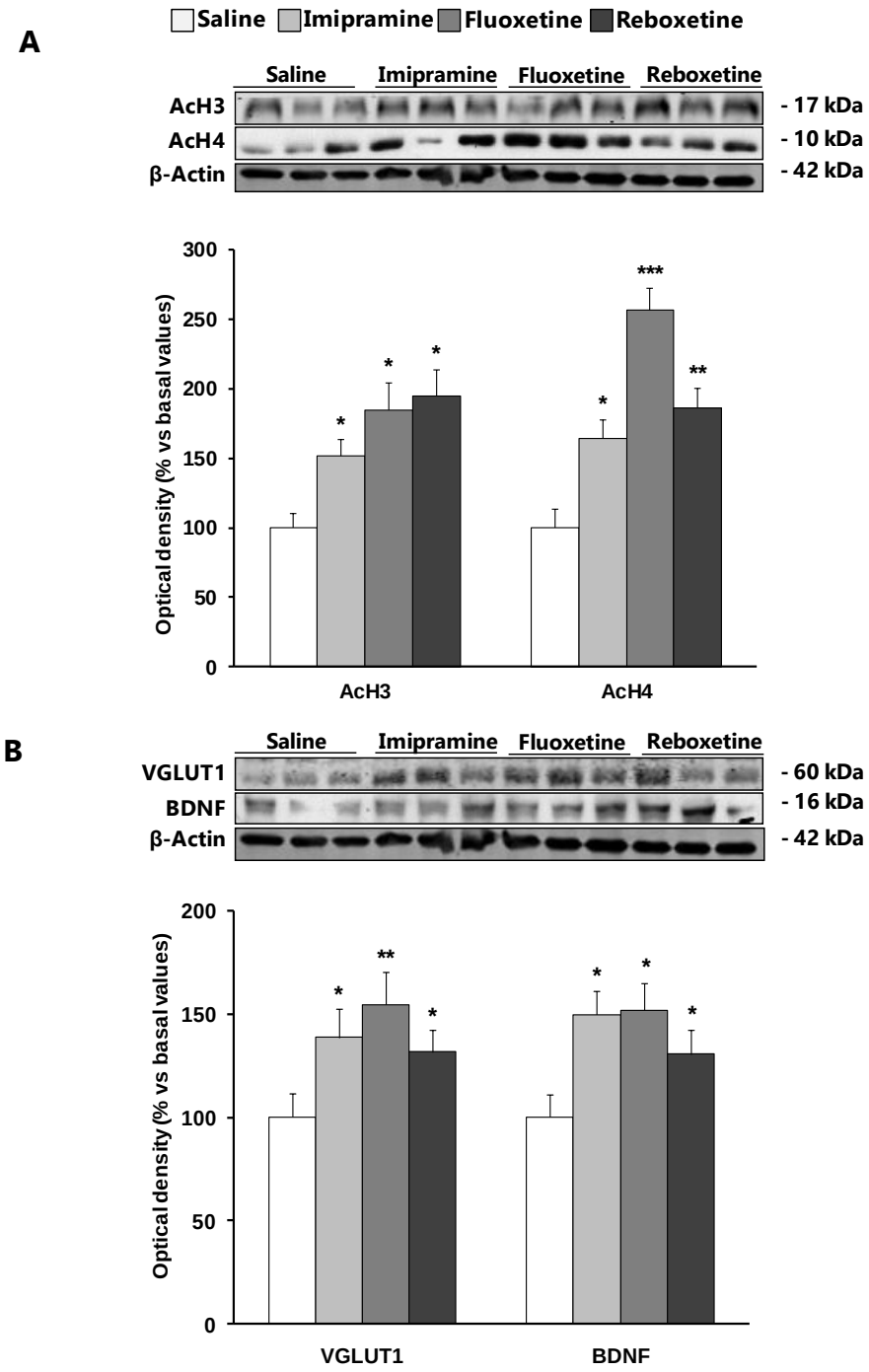


Figure 19. Antidepressants increase histone acetylation and synaptic plasticity markers *in vivo*. (A) Effect of chronic treatment with imipramine (10 mg/kg), fluoxetine (15 mg/kg) and reboxetine (15 mg/kg) for three weeks on AcH3 and AcH4 expression and (B) on VGLUT1 and BDNF expression in mice prefrontal cortex. Values show the mean ± SEM of relative abundance of protein expression compared to controls. ***p<0.001, **p<0.01, *p<0.05 *vs* corresponding controls. One-way ANOVA followed by Dunnett test. Abbreviations: AcH3, acetylated histone 3; AcH4, acetylated histone 4; VGLUT1, vesicular glutamate transporter 1; BDNF, brain-derived neurotrophic factor.

2. Therapeutic potential of SIRT2 for depression treatment in the VGLUT1+/- model

The second aim of our study was to evaluate the therapeutic potential of SIRT2 for depression treatment. Specifically, the potential antidepressant effect of the selective SIRT2 inhibitor 33i was tested in mice heterozygous for VGLUT1 (VGLUT1+/-), previously characterized as a genetic model of depressive-like behavior. Firstly, we tested whether the VGLUT1+/- model shows alterations in the expression of the HDAC superfamily or in histone acetylation in the PFC. Subsequently, the effect of 33i and reference antidepressant imipramine in the anhedonic behaviour of VGLUT1+/- mice was studied. Finally, we tested the activity of 33i for specific monoaminergic molecular targets in order to discard classic antidepressant mechanisms.

2.1. The mRNA expression of the HDAC superfamily in the PFC of VGLUT1+/- mice and wild type littermates

In this experiment we have studied the mRNA abundance of the Hdac's superfamily including class I (*Hdac1, Hdac2, Hdac3* and *Hdac8*), class II (*Hdac4, Hdac5, Hdac6, Hdac7, Hdac9* and *Hdac10*) and IV (*Hdac11*) as well as the NAD^+-dependent class III (*Sirt1-7*) in the PFC of VGLUT1+/- mice, as a genetic model of depression (Garcia-Garcia *et al.* 2009). No alterations in the mRNA expression of any of these enzymes were found, indicating that the depressive-like behavior of this model is not linked to variations in Hdac mRNA expression (Figure 20). Yet, further studies should confirm these results at protein level.

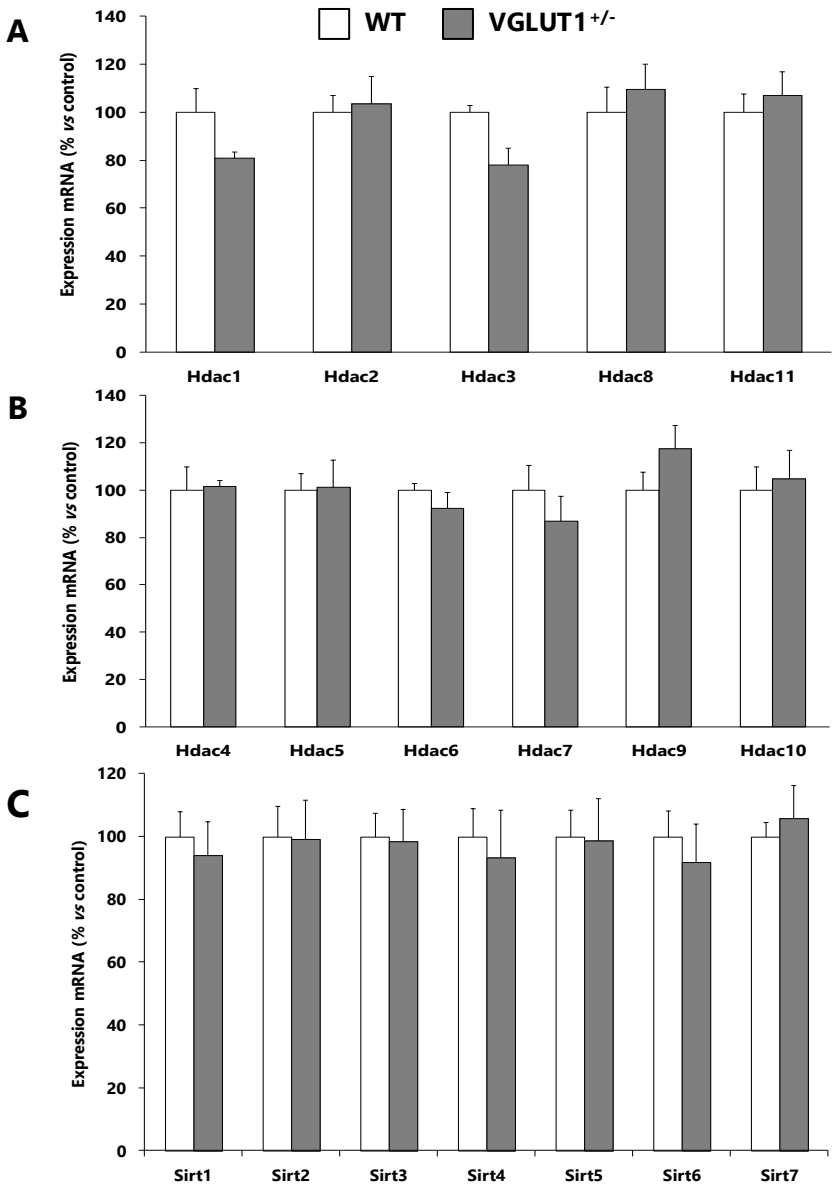


Figure 20. Hdac superfamily is not altered in the PFC of VGLUT1+/- mice. mRNA abundance of histone deacetylase enzymes (Hdac's) (A) Hdac's class I and IV, (B) class II and (C) class III in mice prefrontal cortex of VGLUT1+/- mice and WT littermates. Data show average ± SEM of relative abundance of mRNA expression compared to WT values (Student t-test, n=8-9 mice/group).

2.2. VGLUT1+/- mice show no changes in histone acetylation, HDAC5 and SIRT2

Subsequently, we tested the therapeutic value of SIRT2 inhibition on HDAC5, p-HDAC5 (Figure 21A), SIRT2, acetylated α-tubulin (Figure 21B) AcH3 and AcH4 (Figure 21C). We observed that the protein levels of AcH3 and AcH4 were not altered in the

PFC of VGLUT1+/- mice compared to WT littermates. Similarly, no changes were observed either in HDAC5, p-HDAC5, SIRT2 and acetylated α-tubuline. As expected VGLUT1 expression levels were significantly ($p<0.05$) reduced in the mouse PFC of VGLUT1+/- mice compared to WT littermates (Figure 21D). No effects on the expression levels of all these proteins were observed.

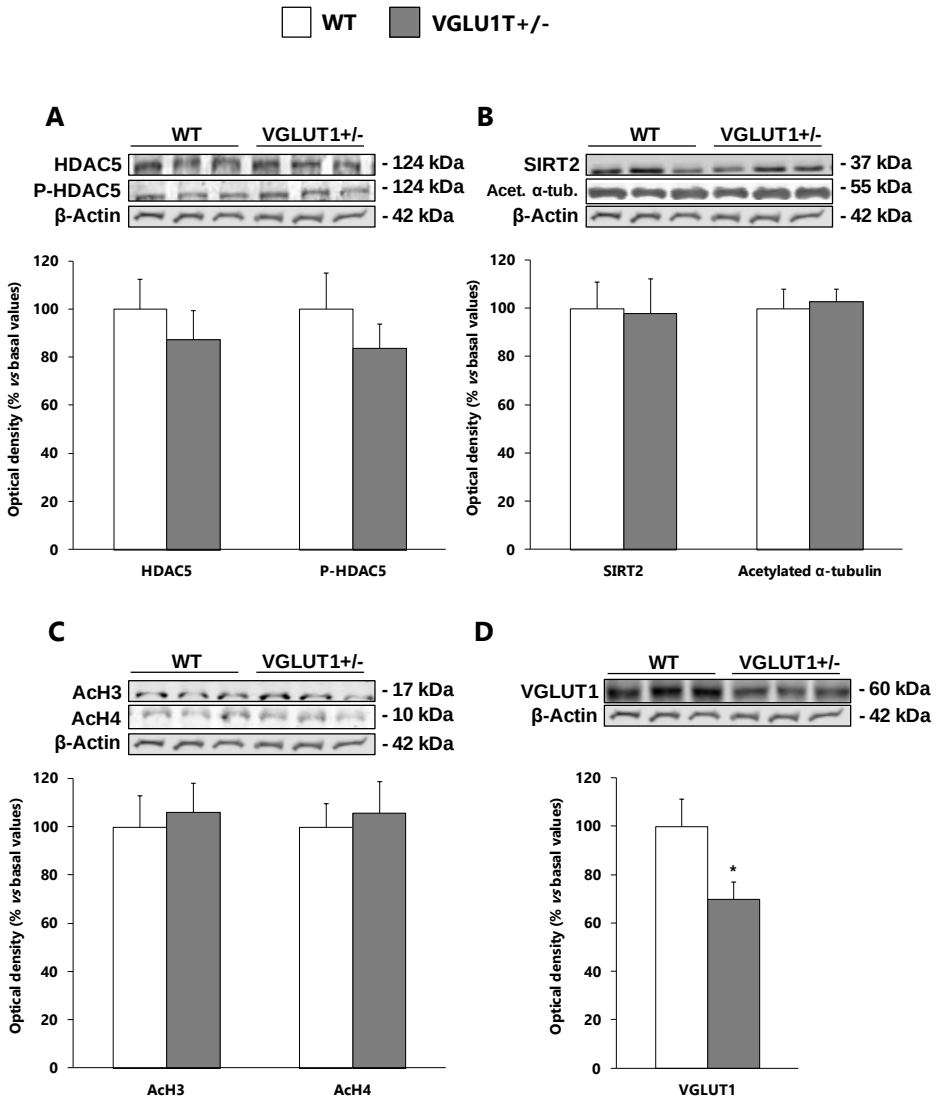


Figure 21. Epigenetic targets expression in the VGLUT1+/- mice model. ffect of 2 hour incubation with antidepressants on histone acetylation, VGLUT1 and BDNF *in vitro*. Protein expression of (A) HDAC5 and p-HDAC5, (B) SIRT2 acetylated α-tubulin, (C) AcH3 and AcH4 and (D) VGLUT1 expression. Values show the mean ± SEM of relative abundance of protein expression compared to controls. *$p<0.05$ *vs* corresponding WT littermates (Student t-test, n=8-9 mice/group). Abbreviations: HDAC5, histone deacetylase 5; p-HDAC5, phospho-histone deacetylase; SIRT2, sirtuin 2; AcH3, acetylated histone 3; AcH4, acetylated histone 4; VGLUT1, vesicular glutamate transporter 1.

2.3. The SIRT2 inhibitor 33i reverses anhedonia in the VGLUT1+/- mice model

In this experiment, we tested the therapeutic value of SIRT2 inhibition in the anhedonic behaviour of VGLUT1+/- mice. Firstly, we examined the effect of imipramine as a reference antidepressant drug on anhedonic behaviour of VGLUT1+/- mice (Figure 22A). While no interaction among time (weekly repeated measures), treatment and genotype on sucrose intake was found [$F_{4,256}$= 0.65 p>0.05], two-way ANOVA for each week presented a significant main effect of genotype on sucrose intake [$F_{1,64}$= 21.25, 10.9, 9, 9.7 $p < 0.01$, for weeks 1, 2, 3 and 5, respectively]. Similarly, a significant main effect of imipramine treatment was found at weeks 4 and 5 [$F_{1,64}$= 4.1, $p < 0.05$ and 11.5, $p < 0.01$, respectively]. Thus, VGLUT1+/- mice showed a decrease in sucrose intake that would be indicative of anhedonia. On the other hand, imipramine increased significantly sucrose intake showing an antianhedonic action from the fourth week until the end of treatment (Figure 22A).

Subsequently, the effect of the 33i compound on anhedonic behaviour of VGLUT1+/- mice was tested. Again, no interaction among time (weekly repeated measures), 33i treatment and genotype was found [$F_{4,252}$= 1.24 p>0.05] on sucrose intake. Two-way ANOVA for each week showed a significant main effect of genotype on sucrose intake [$F_{1,63}$= 11.1, 9.3 and 10.3, $p < 0.01$, for weeks 1, 2 and 3, respectively]. Moreover, a significant interaction between genotype and 33i treatment was found at weeks 4 and 5 [$F_{1,63}$= 7.8 and 8.1, $p < 0.01$, respectively]. *Post hoc* analysis revealed that 33i treatment was able to reverse anhedonic behaviour exhibited by VGLUT1+/- mice [$F_{3,63}$ = 9.1 and 7.9, $p < 0.01$, respectively for weeks 4 and 5] (Figure 22B).

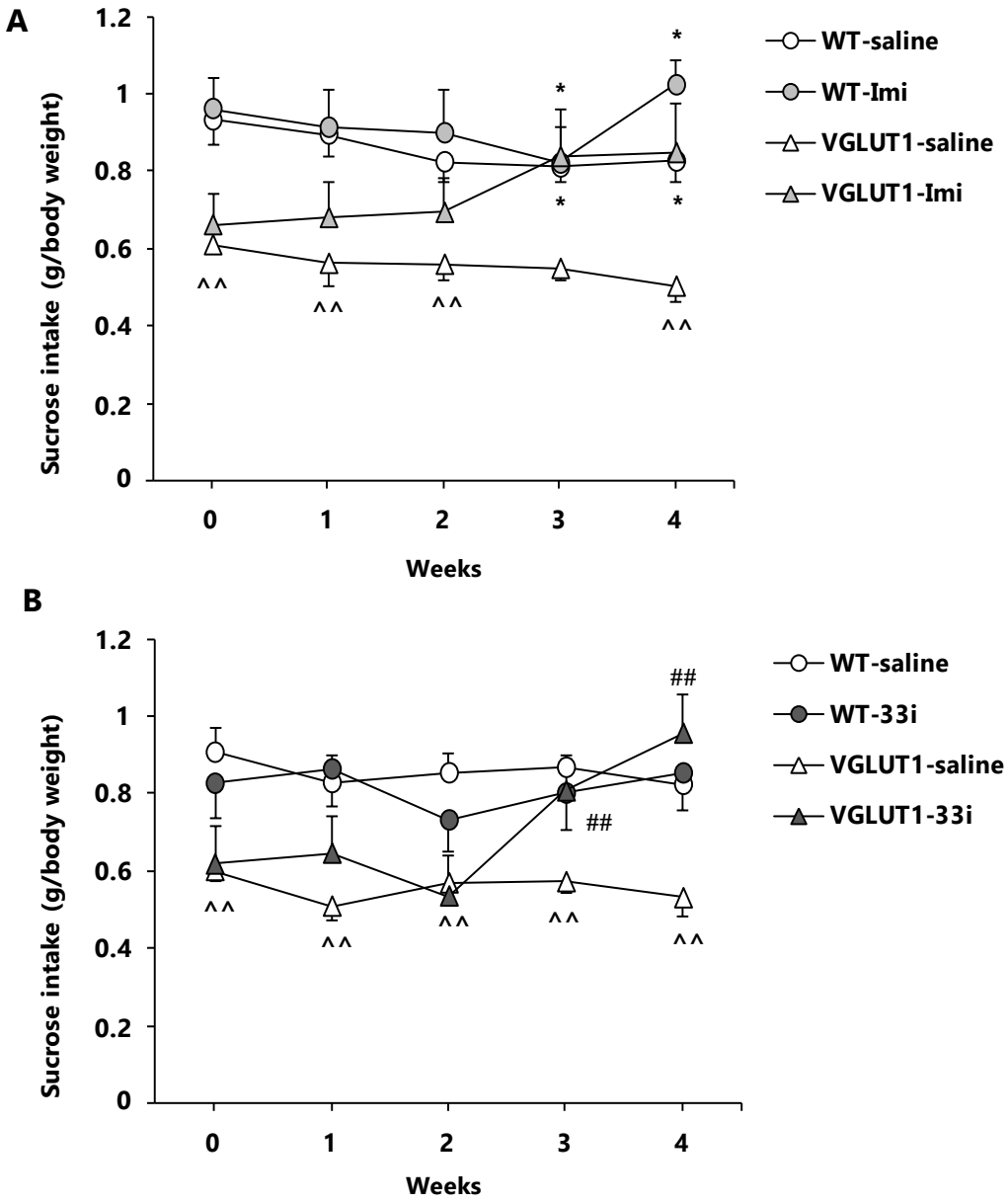


Figure 22. SIRT2 inhibition rescues anhedonic behaviour of VGLUT1+/- mice. Effect of (A) imipramine and (B) 33i treatment on depressive like behaviour in the sucrose intake test. Data show mean ± SEM of different groups. *p<0.05, main effect of treatment; ^^p<0.01 main effect of genotype; ##p<0.01 VGLUT1+/- treated with 33i *vs* VGLUT1+/- treated with saline (two way ANOVA followed by Tukey-post hoc, n= 15-20 mice per group).

2.4. Study on the activity of the SIRT2 inhibitor 33i towards specific monoaminergic molecular targets for depression

We further studied here whether 33i interacts with specific monoaminergic targets for depression treatment aside from its ability to inhibit SIRT2. Specifically, while the reference compounds fluoxetine and reboxetine at 10 μM inhibited effectively [^{3}H]paroxetine binding (82±2.5%) and [^{3}H]noradrenaline synaptosomal uptake (65±2.6%) respectively, the compound 33i (10 μM - 1 nM) showed no affinity for the 5-HT transporter or inhibitory activity over [^{3}H]-noradrenaline uptake (data not shown). Moreover, it was shown in table 2, 33i (10 μM - 1 nM) had no effect on monoaminooxidase (MAO) enzyme activity.

Table 2. Lack of activity of 33i over MAO enzyme. Specific activity (μU/mg of protein) of monoamino oxidase enzyme in the presence of clorgyline (10 μM), selegiline (10 μM) and 33i (0.01 μM-100 μM).

	Concentration (μM)	Specific activity MAO (μU/mg of protein)	% MAO-total inhibition
Vehicle	-	44.2	
Clorgyline (MAO-A inhibitor)	10	21.3	51.8
Selegiline (MAO-B inhibitor)	10	31.5	28.7
33i	0.01	45	-1.02
	1	41.2	6.78
	100	44.4	-0.45

Unit definition: one unit of MAO catalyzes the formation of 1 μmole H_2O_2 per min under the assay conditions

3. Role of induced expression of VGLUT1 in the PFC in antidepressant action

Here we have initiated a study directed to examine the role of the PFC VGLUT1 in the long-loop mechanisms of control of 5-HT system as well as in the modulation of depressive-like behaviors. Firstly, an adeno-associated virus (AAV) expressing the VGLUT1 gene was designed and its functionality was evaluated. Subsequently, the pAAV-pSyn-VGLUT1-mCherryminisog-SMD2 was injected in the PFC of mice and the optimal concentration required for imaging VGLUT1^{mCherryminisog} expression both in the PFC and in glutamatergic descending fibbers in the dorsal raphe nucleus (DRN) was determined. Finnally, using the VGLUT1+/- model, we have carried out preliminary studies directed to evaluate the effect of induced VGLUT1 expression in the PFC on depressive-like behaviour as well as on 5-HT$_{1A}$ function have been carried out.

3.1. Functionality of the generated plasmid pAAV-pSyn-VGLUT1-mCherryminisog-SMD2 in rat hippocampal cultures

The mCherryminisog protein is bright red fluorescent and was visible *in vitro* for in rat hippocampal cultures transfected with the pAAV-pSyn-VGLUT1mCherryminisog-SMD2. VGLUT1^{mCherryminisog} was effectively driven by the synapsin promoter and expressed in hippocampal neurons (Figure 23).

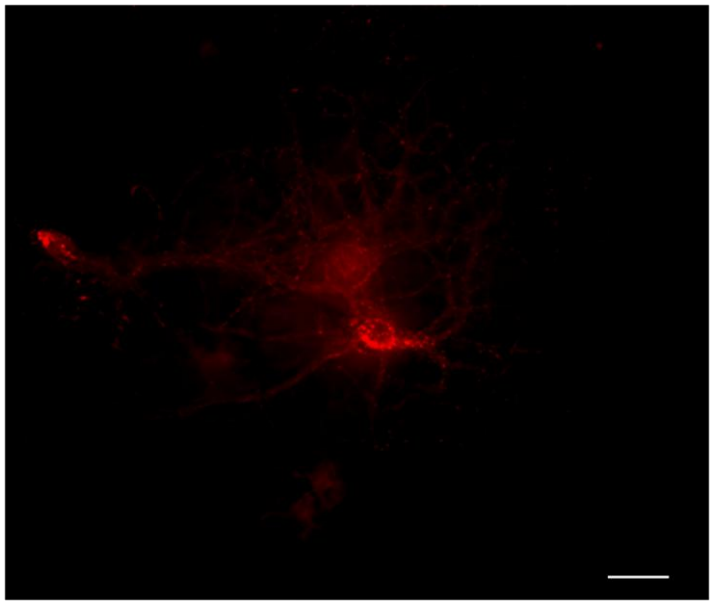


Figure 23. pAAV-pSyn-VGLUT1-mCherryminisog-SMD2 functionality Hippocampal rat neurons two weeks after electroporation 1:10,000 dilution of pAAV-pSyn-VGLUT1^{mCherryminisog}. Scale bar means 20 µm.

3.2. pAAV-pSyn-VGLUT1^{mCherryminisog} and pAAV-pSyn-YFP imaging in the PFC, in the DRN and other projecting areas on VGLUT1+/- mice and WT littermates

In preliminary experiments, of the different dilutions tested, for AAV-pSyn-VGLUT1^{mCherryminisog} (1:40, 1:16, 1:4, 1:2 and 1:1), the 1:2 and 1:1 dilutions imaging studies revealed VGLUT1^{mCherryminisog} expression in both cell bodies and fibbers in the PFC as well as in fibbers in the dorsal raphe nucleus (DRN). Yet, using lower dilutions (1:4-1:40) of the AAV, no signal at the level of the DRN was detected. Therefore the 1:2 dilution was selected for subsequent studies.

Hence, *in vivo* gene transfer efficacy was determined by imaging analysis using the epifluorescence microscope (4x, 40x). Fluorescence was visible in serial sliced sections of the PFC of mouse brain injected with pAAV-pSyn-VGLUT1^{mCherryminisog} under synapsin promoter. Abundant ectopic VGLUT1 could be detected specifically in the mPFC of pAAV-pSyn-VGLUT1^{mCherryminisog}, including infralimbic (Figures 24A) and other cortical areas (data not shown). The rostro-caudal extension of the vector spread was observed in all the slices around the injection site. At higher magnification (40X) ectopic VGLUT1 expression was visualized in neuronal soma and the neuropil (Figures 24A'). Further, analysis of mCherryminisog or YFP fluorescence in the whole PFC of both genotypes, WT and VGLUT1+/-, revealed no differences in terms of area by intensity of fluorescence (Figures 24C and 24C').

The YFP protein is bright green fluorescent. pAAV-pSyn-YFP injected animals showed fluorescence in serial sliced sections of the PFC (Figure 24B and 24B').

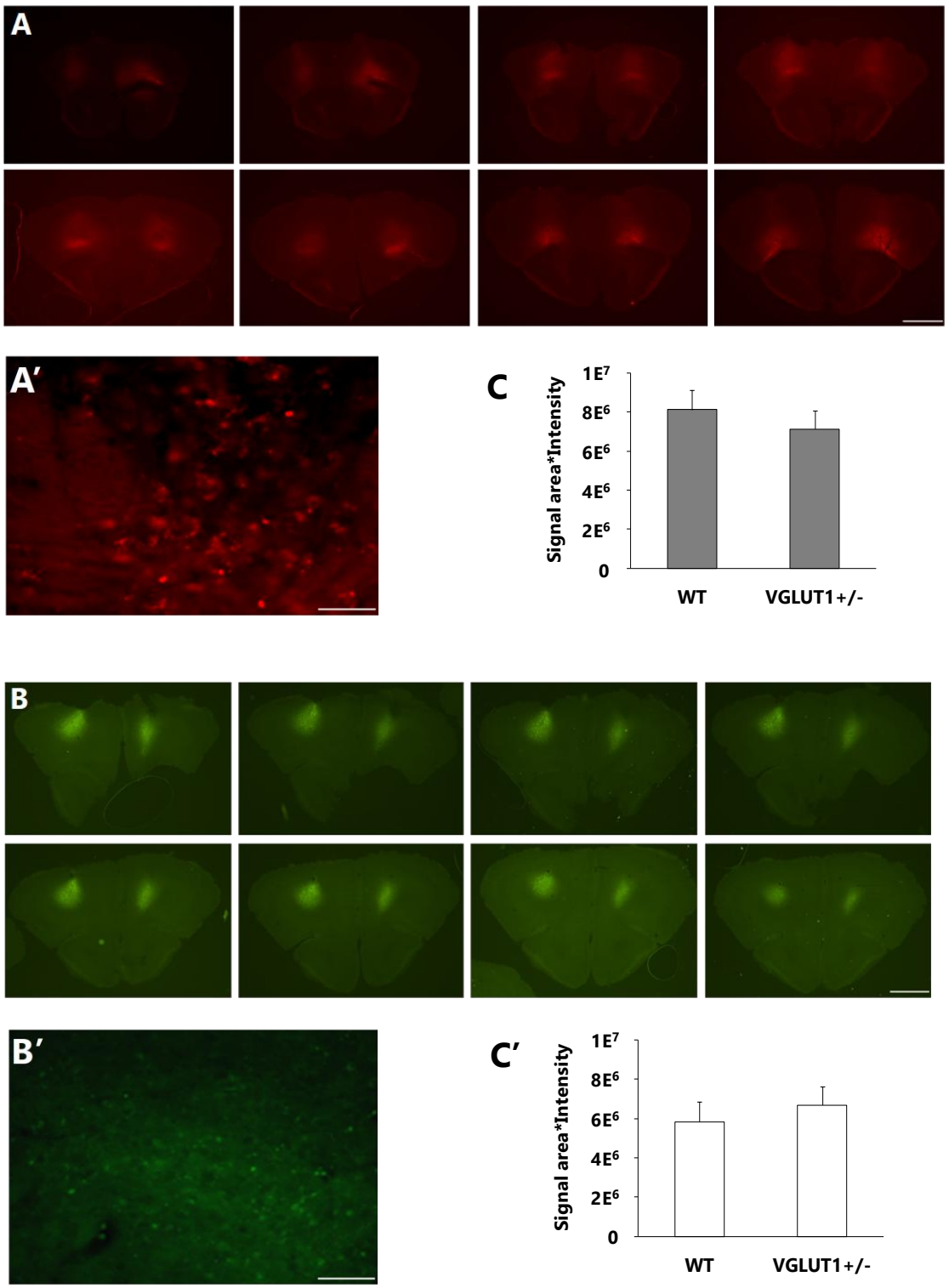


Figure 24. Adeno-associated (AAV) virus-mediated VGLUT1 expression in the PFC-IL (A-B) Representative expression of the PFC of mice injected with (A) pAAV-pSyn-VGLUT1^{mCherryminisog} and (B) pAAV-pSyn-YFC at (A-B) low 4X and (A'-B') high 40X magnification. (C-C') Quantification of AAV efficiency (score signal in the area * intensity) in the PFC of WT and VGLUT1+/- mice. In A and B scale bar means 1 mm and in A'-B' means 50 µm.

In the dorsal raphe nucleus (DRN), we observed ectopic VGLUT1 expression in fibbers (Figure 25A). In addition, signal of YFP fluorescence was detected in this area showing that the injected point selected has projections to this area (Figure 25B). In addition at the level of the dorsal hippocampus, VGLUT1 immunoreactivity could be observed in hypothalamic areas, in the amygdala and in the dentate girus of the hippocampus (Figure 26).

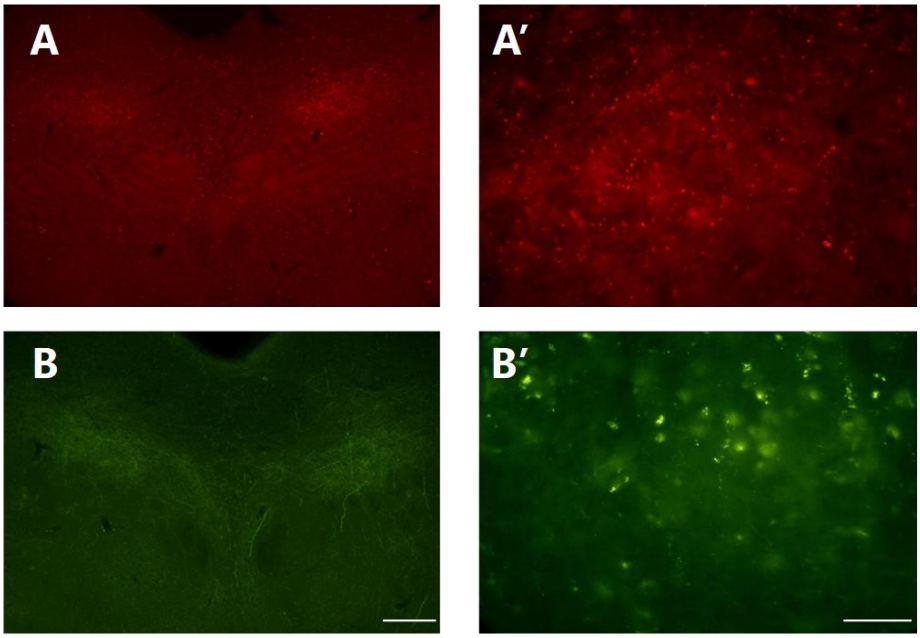


Figure 25. **Adeno-associated (AAV) virus-mediated VGLUT1 expression in the in the raphe nuclei.** Representative expression of the raphe nuclei of mice injected with (A) pAAV-pSyn-VGLUT1^{mCherryminisog} and (B) pAAV-pSyn-YFC at (A-B) low 10X and (A'-B') high 40X magnification. In A and B scale bar means 500 µm and in A'-B' means 50 µm.

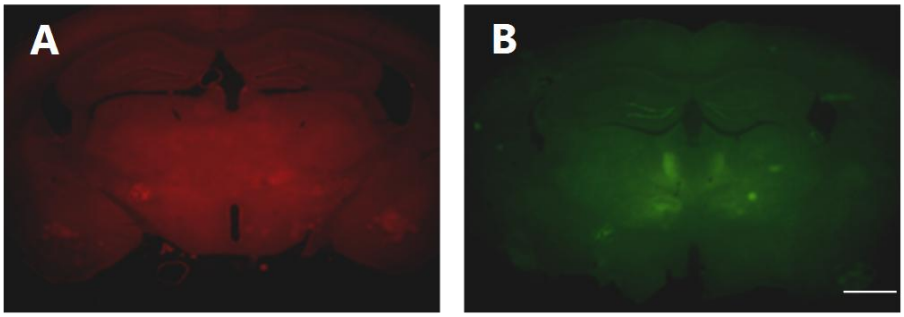


Figure 26. **Adeno-associated (AAV) virus-mediated VGLUT1 expression in PFC projecting areas.** Representative expression of the Hippocampus and the amygdale of mice injected with (A) pAAV-pSyn-VGLUT1^{mCherryminisog} and (B) pAAV-pSyn-YFC at 4X magnification. Scale bar means 1 mm.

3.3. VGLUT1-induced expression in the PFC is able to rescue the anhedonic-like behaviour of VGLUT1+/- mice

Two-way ANOVA repeated measures showed that mice weight gain was affected across the seven weeks of the experiment [$F_{4,140}$= 24.2, p<0.001]. In general there was a weight gain between 0.4 and 0.9 g/week. But on week 1, corresponding to 7 days following stereotaxic viral injections, one-way ANOVA showed a significant decrease (-0.27±0.10 g) (p<0.001) on weight gain [$F_{4,190}$= 28.39, p<0.001] compared to the rest of weeks (week 0, 2, 3 and 7) (Figure 27A).

Repeated measures two-way ANOVA with genotype and AAV injection as between-subject and time (weeks) as within subject factors revealed a significant interaction between AAV treatment and time on sucrose intake [$F_{4,140}$= 3.72; p<0.01]. For each week, two-way ANOVA of individual measures revealed that VGLUT1+/- mice showed lower sucrose intake compared to WT mice in all weeks ($F_{1,35}$= 9.69, 9.90, 21.52, 6.88, 10.32, for weeks 0, 1, 2, 3 and 7 respectively; p<0.01, main effect of genotype]. In addition, pAAV-pSyn-Vglut1^{mCherryminisog} treatment increased sucrose intake [$F_{1,35}$= 21.86, 50.35, 15.28, 25.86, for weeks 1, 2, 3 and 7 respectively; p<0.01, main effect of pAAV-pSyn-Vglut1^{mCherryminisog} treatment] (Figure 27B).

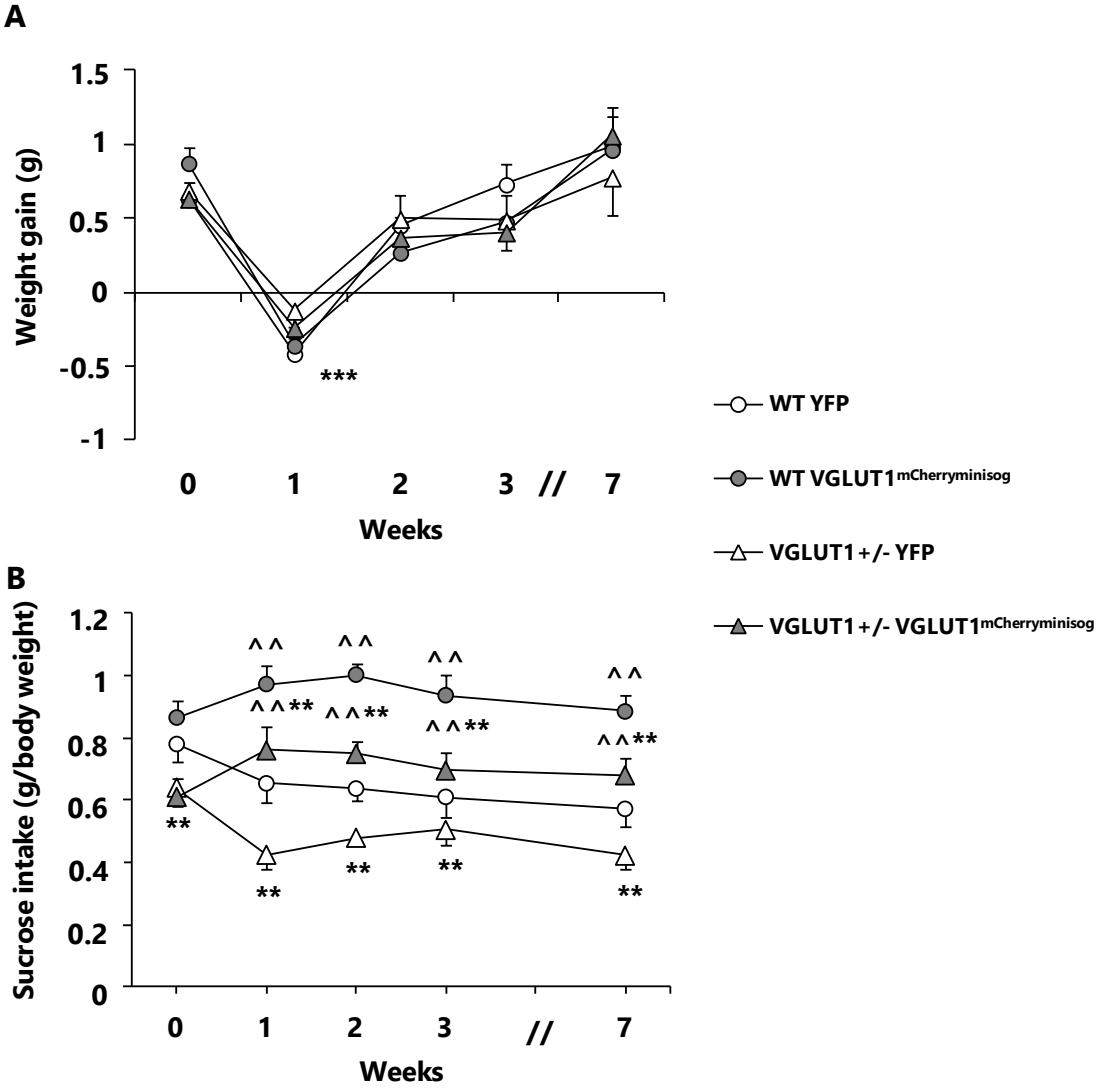


Figure 27. Effect of VGLUT1-induced expression in the IL-PFC on body weight gain (A) and sucrose intake (B) of WT and VGLUT1+/- mice. Data show the mean ± SEM (n= 9-11 mice/ group) of the weekly body weight gain (g) (A) and sucrose intake (g per body weight). **p<0.01, main effect of genotype; ^^p<0.01 main effect of pAAV-pSyn-VGLUT1^{mCherryminisog} treatment (Two- way ANOVA).

3.4. Behavioural studies

Locomotor Activity. WT and VGLUT1 mice treated with either pAAV-pSyn-VGLUT1^{mCherryminisog} or pAAV-pSyn-YFP did not differ in their spontaneous locomotor activity during the 30-minutes test period (Figure 28A).

Open field. In the open field test, stereotaxic injection of pAAV-pSyn-VGLUT1^{mCherryminisog} decreased significantly time spent in center zone in both WT and VGLUT1+/- mice [$F_{1,35}$= 5.59, $p<0.05$] (Figure 28B). However no differences in latency time to go to the center zone were observed among the groups (data not shown).

Social Interaction Test. No significant differences among the groups (WT YFP, WT VGLUT1^{mCherryminisog}, VGLUT1+/- YFP and VGLUT1+/- VGLUT1^{mCherryminisog}) were observed in interaction time [$F_{1,35}$= 1.34, $p>0.05$] (Figure 28C). In addition freezing times were not altered [$F_{1,35}$= 0.18, $p>0.05$] (data not shown).

Marble burying test. In the marble burying test, stereotaxic injection of pAAV-pSyn-VGLUT1^{mCherryminisog} decreased significantly the number of buried marbles in both WT and VGLUT1+/- mice [$F_{1,35}$= 6.01, $p<0.05$] (Figure 28D).

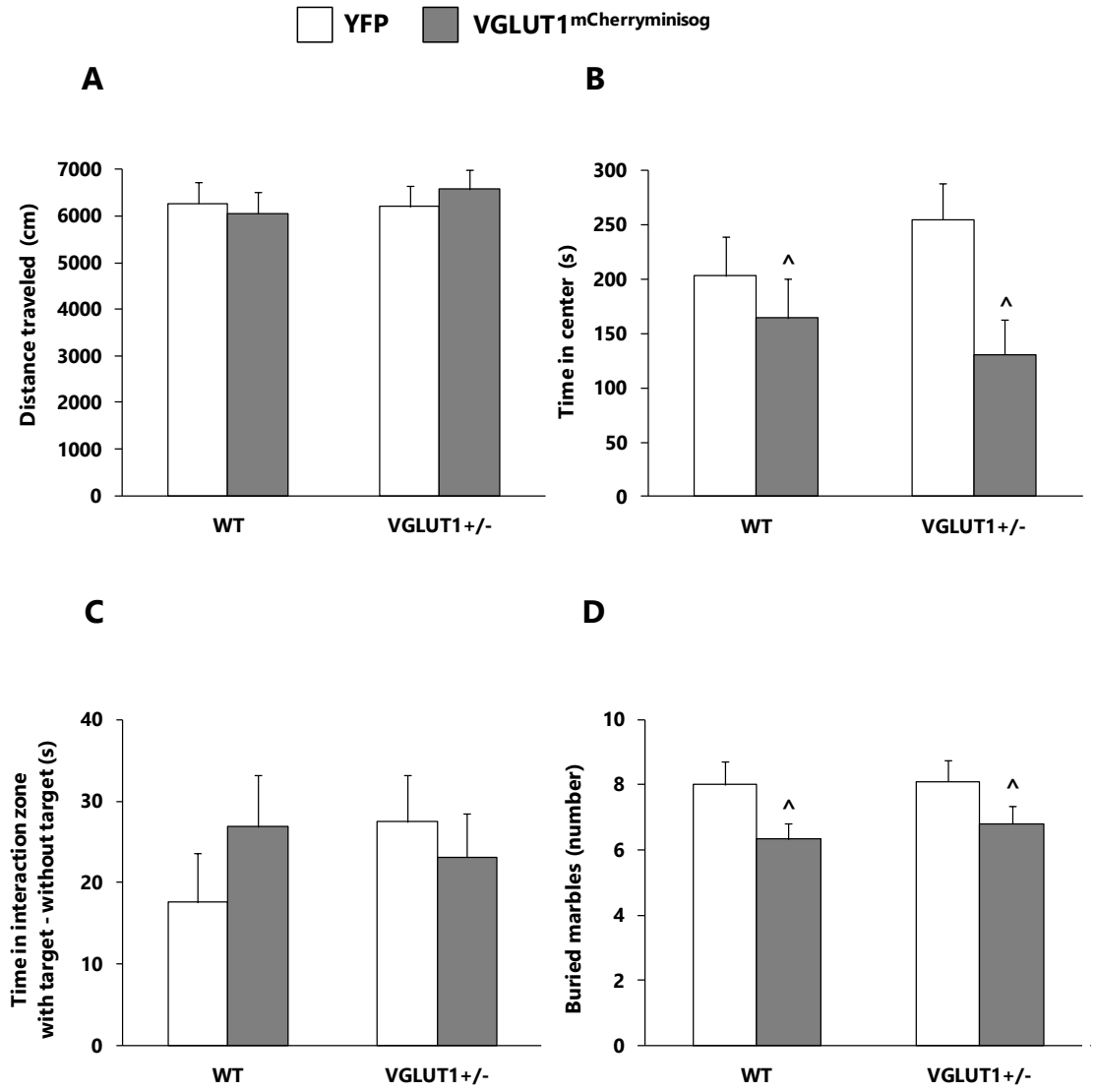


Figure 28. Effect of VGLUT1-induced expression in the IL-PFC on spontaneous locomotor activity (A), open field (B), marble buying (C) and social interaction (D) tests of WT and VGLUT1+/- mice. Data show the mean ± SEM (n= 9-11 mice/ group) of distance travelled (A), time in center zone (B), difference of time spent in interaction zone in the presence and the absence of a target (C) and the number of marbles buried (D). ^^p<0.01 main effect of pAAV-pSyn-VGLUT1^{mCherryminisog} treatment (Two-way ANOVA).

3.5. Effect of VGLUT1-induced expression in the PFC on the hypothermic response mediated by a 5-HT$_{1A}$ agonist

Hypothermia induced by the 5-HT$_{1A}$ agonist 8-OH-DPAT was studied in WT and VGLUT1+/- mice as an *in vivo* measure of presynaptic 5-HT$_{1A}$ autoreceptors function in the raphe nucleus (Bill *et al.* 1991). Baseline temperature values were similar in both WT

(36.9±0.26ºC) and VGLUT1+/- (36.7±0.22ºC) mice. Subcutaneous 8-OH-DPAT injection induced a time dependent decrease of body temperature [$F_{2,64}$= 42.55, p<0.001]. Significant interactions between genotype (VGLUT1+/- *vs* WT) and treatment (pAAV-pSyn-VGLUT1^{mCherryminisog} *vs* pAAV-pSyn-YFP) in the hypothermic response induced by 8-OH-DPAT were observed at 30, 60 and 90 min post injection [$F_{1,32}$= 6.36, 5.87 and 6.53; p<0.05]. One-Way Anova for each time point revealed that at 30 minutes, 8-OH-DPAT injection induced a lower decrease of body temperature (-0.85±0.11ºC) in VGLUT1+/- pAAV-pSyn-YFP injected mice compared to WT pAAV-pSyn-YFP injected littermates (-2.0±0.31ºC). On the other hand, VGLUT1+/- mice injected with pAAV-pSyn-VGLUT1^{mCherryminisog} showed an 8-OH-DPAT induced hypothermia (-1.96±0.27ºC) similar to WT pAAV-pSyn-YFP injected littermates. At 60 min after 8-OH-DPAT injection, VGLUT1+/- mice injected with the pAAV-pSyn-VGLUT1^{mCherryminisog} showed a similar 8-OH-DPAT induced hypothermia (-1.5±0.0.26ºC) compared to WT pAAV-pSyn-YFP injected littermates (-1.31±0.52ºC) (Figure 29).

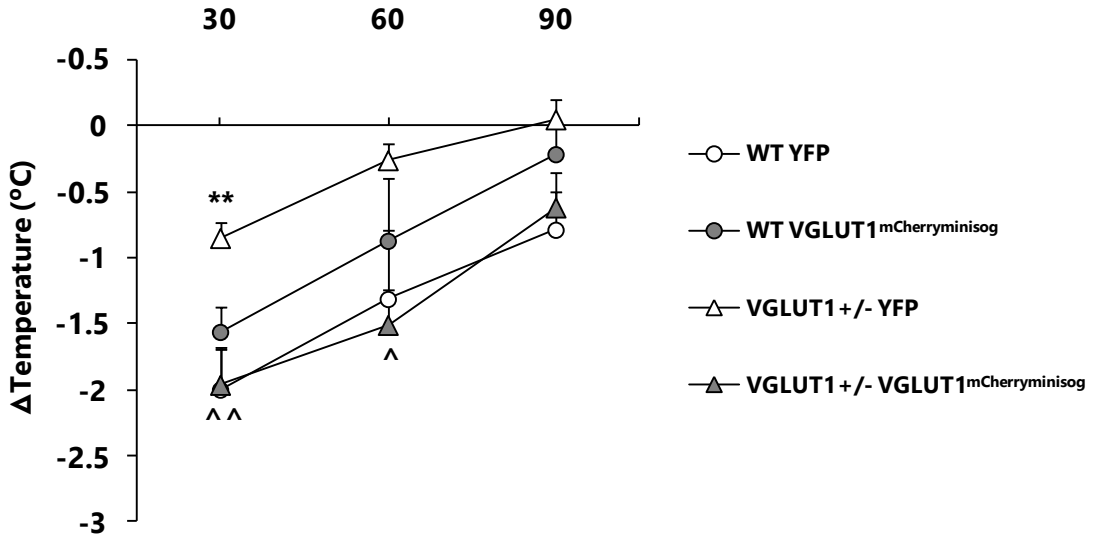


Figure 29. Effect of VGLUT1-induced expression in the IL-PFC on the hypothermic response to 8-OH-DPAT (0.4 mg/kg, s.c.) at different times, in VGLUT1+/- and WT mice. One-way ANOVA revealed effect of genotype and pAAV-pSyn-VGLUT1^{mCherryminisog} treatment. Values are expressed as mean ± SEM (**p<0.01 and *p<0.05 *vs* WT; ^^p<0.01 and ^p<0.05 *vs* VGLUT1+/- YFP control).

3.6. Age-dependent 5-HT$_{1A}$ mRNA expression in VGLUT1+/- mice and WT littermates

As it can be observed in figure 30, no changes in 5-HT1A mRNA expression were observed in VGLUT1+/- mice compared to WT littermates, at any of the ages (P7, P21 and P35) tested.

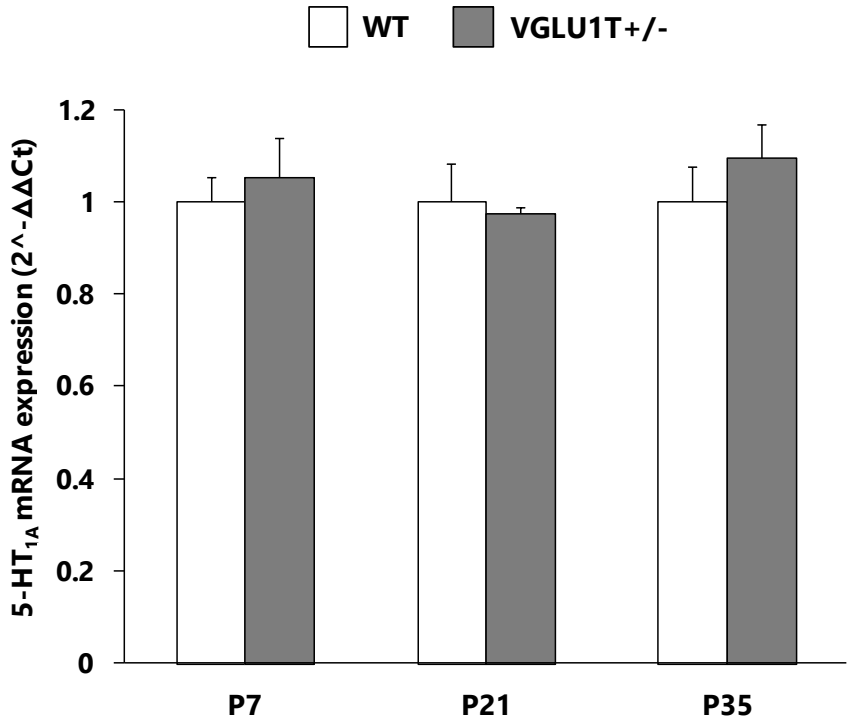


Figure 30. Age-dependent 5-HT$_{1A}$ mRNA expression. No chages in mRNA abundance of 5-HT$_{1A}$ autoreceptor in mice brain of VGLUT1+/- mice and WT littermates. Data show average ± SEM of relative abundance of mRNA expression compared to WT values (Student t-test, n=8-9 mice/group).

DISCUSSION

1. Nucleocytoplasmic export of HDAC5 and SIRT2 downregulation: two epigenetic mechanisms by which antidepressants enhance synaptic plasticity markers

This study presents two epigenetic mechanisms, by which antidepressants could enhance synaptic plasticity markers linked to antidepressant action. Briefly, the three antidepressants tested (fluoxetine, reboxetine and imipramine) regulated differently the histone deacetylases (HDACs) HDAC5 and SIRT2. Particularly, imipramine and reboxetine increased the phosphorylated form of HDAC5 (p-HDAC5), indicating that noradrenaline mediates cytoplasmic export of this enzyme. On the other hand, the three antidepressants downregulated SIRT2. Specific inhibition of HDAC5 with MC3822 (Bürli *et al.* 2013) and SIRT2 with 33i compound (Suzuki *et al.* 2012) increased synaptic plasticity markers too. Finnally, as expected, antidepressant treatment increased synaptic plasticity markers and histone acetylation. Thus, nucleocytoplasmic export of HDAC5 and SIRT2 downregulation mediated by antidepressants could enhance synaptic plasticity markers leading to antidepressant action.

1.1. Nucleocytoplasmic export of HDAC5 by antidepressants enhance synaptic plasticity markers

Growing evidence suggests that changes in histone acetylation are important posttranslational regulators of gene expression and therefore may play a key role in neuronal plasticity (Abel and Zukin 2008; Dulac 2010). Acetylation lowers the affinity between histones and DNA allowing chromatin to adopt a more relaxed structure and favoring transcription. Deacetylation, mediated by HDACs, is generally associated with transcriptional repression (Gallinari *et al.* 2007).

The HDAC5 enzyme belongs to the class IIa protein superfamily (HDAC4, 5, 7 and 9). This class suffers synaptic activity-dependent nucleo-cytoplasmic shuttling (McKinsey *et al.* 2001; Chawla *et al.* 2003; Renthal *et al.* 2007; Erburu *et al.* 2015a). The phosphorylated form in turn shuttles out of the nucleus, allowing the expression of its target genes (Parra and Verdin 2010). HDAC5 phosphorylates at S259 and S498, favouring its cytoplasmic localization (Vega *et al.* 2004).

In the current study, we found that the tricyclic imipramine and the selective noradrenaline reuptake inhibitor reboxetine increased the content of the phosphorylated form of HDAC5 (p-HDAC5) *in vitro* in cell cultures. On the other hand, the selective serotonin reuptake inhibitor fluoxetine did not affect to HDAC5 phosphorylation state. Therefore, it is suggested that HDAC5 phosphorylation is mediated specifically by increased levels of noradrenaline in the synaptic cleft, a mechanism shared by imipramine and reboxetine. Interestingly, a role for α_1 noradrenaline receptor in the phosphorylation and export of HDAC5 to the cytoplasm has been described (Sucharov *et al.* 2011).

Supporting western-blot results, the nucleocytoplasmic shuttling induced by noradrenaline was confirmed by immunofluorescence in cell cultures. Specifically, reboxetine induced a striking increase of HDAC5 immunoreactivity in the cytoplasm of cultured neurons, while a decrease of this enzyme was found in the nucleus.

Interestingly, at 2 h incubation time, reboxetine increased p-HDAC5 suggesting that nuclear export of HDAC5 is a rapid-acting effect mediated by noradrenaline that would contribute to histone acetylation. In keeping with this, nuclear export of HDAC5, 4 hours after cocaine (Renthal *et al.* 2007) and 3-6 hours after ketamine (Choi *et al.* 2015) has been shown.

Comparatively, chronic imipramine and reboxetine upregulated p-HDAC5 *in vivo* in the PFC of mice, a result that agrees with a previous study carried out in our lab (Erburu *et al.* 2015a). Altogether, these findings suggest that both antidepressants attenuated HDAC5 repressive influence on transcription by inducing the nuclear export of HDAC5 to the cytoplasm. Matching with this, Bdnf promoter has been reported to be controlled by HDAC5 (Tsankova *et al.* 2006).

Subsequently, we tested two enantiomers (R,R,R and S,S,S) of a new selective HDAC5 inhibitor (MC3822 an MC3823 respectively). Between the two and matching with its HDAC4/5 inhibitory activity, MC3822 was found to increase more effectively AcH3 and AcH4 and was selected as the active enantiomer. Subsequently, MC3822 was selected to study the specific inhibition of HDAC5 on neuroplasticity. Particularly, the synaptic plasticity markers the brain derived neurotrophic factor (BDNF) and the

vesicular glutamate transporter 1 (VGLUT1) were selected.

The neurotrophin hypothesis of depression postulates that BDNF is involved in depression as well as in the clinical response to antidepressants. It stimulates growth, differentiation and survival of neurons and its expression is oppositely regulated by stress and antidepressant treatment (Alonso *et al.* 2004; Czéh *et al.* 2007; Duman and Monteggia 2006). In patients, while low levels of brain and serum BDNF have been associated to major depression (Nestler *et al.* 2002), antidepressant treatment boosts BDNF (Duman 2014).

Besides neurotrophins, VGLUT1 has been also associated to depression and antidepressant action. On the whole, VGLUT1 is the predominant isoform of excitatory terminal of telencephalic regions (Fremeau *et al.* 2001; Gras *et al.* 2002) having a key role on synaptic release of glutamatergic transmission (Wojcik *et al.* 2004; Balschun *et al.* 2010). A course of antidepressant treatment upregulate VGLUT1 expression in PFC and hippocampus (Tordera *et al.* 2005; Moutsimilli *et al.* 2005; Cooke *et al.* 2014). Interestingly, this effect could be mediated, at least in part, by BDNF (Farley *et al.* 2012). Conversely, decreased VGLUT1 levels in the frontal cortex of depressed subjects have been found (Uezato *et al.* 2009). Moreover, decreased VGLUT1 has been associated to a depressive-like phenotype in rodents (Tordera *et al.* 2007; Garcia-Garcia *et al.* 2009).

MC3822 increased VGLUT1 and BDNF expression indicating that specific inhibition of HDAC5 upregulates neuroplasticity. Additionally, this fact further strengths the idea that the effect of noradrenergic antidepressants on synaptic plasticity could be partially mediated by decreased Hdac5 nuclear function (Tsankova *et al.* 2006; Erburu *et al.* 2015a).

Nevertheless, further behavioural studies should be carried out to establish a causal link between HDAC5 inhibition and antidepressant action. Intriguingly, the potential antidepressant action of HDAC5 inhibition has been recently suggested (Choi *et al.* 2015). For instance, genes involved in cocaine experience-dependent reward sensitivity are silenced by HDAC5 inhibition (Renthal *et al.* 2007). In addition, HDAC5 over-expression blocks antidepressant action in the CSDS model (Tsankova *et al.* 2006). Curiously enough, an upregulation of cortical Hdac5 mRNA has been observed in

subjects with major depressive disorder and bipolar disorder, compared to controls and patients in remission, supporting a clinical relevance for these changes (Hobara *et al.* 2010).

1.2. SIRT2 downregulation by antidepressants enhance synaptic plasticity markers

SIRT2 is a class III histone deacetylase playing a role in transcriptional repression of genes encoding for DNA binding proteins as well as transcription factors that participate in synaptic plasticity, cell proliferation, differentiation and cell survival (Eskandarian *et al.* 2013). Previous findings showing SIRT2 mRNA upregulation in the PFC of depressed patients supports the interest of this target for therapeutic intervention (Erburu *et al.* 2015a). In line with this, preliminary studies carried out in our laboratory have remarkably shown that repeated treatment with the SIRT2 inhibitor 33i causes an antianhedonic action in depression mouse models (Erburu *et al.* 2017).

We observed here that all monoaminergic antidepressants down-regulated the expression of SIRT2 in SH-SY5Y cell cultures suggesting that this could be a shared effect for all antidepressants. Matching with this effect, hyperacetylated α-tubulin levels, a SIRT2 cytoplasmic substrate, were observed. Further *in vivo* studies, revealed again SIRT2 downregulation following chronic antidepressant treatment as previously observed (Erburu *et al.* 2015a). Interestingly, only chronic fluoxetine dowregulated Sirt2 mRNA *in vivo*, suggesting that imipramine and reboxetine act on SIRT2 at post-transductional level.

Furthermore, specific inhibition of SIRT2 by 33i compound also induced an up-regulation of AcH3 and AcH4 as well as VGLUT1 and BDNF in SH-SY5Y cultures. The increase in synaptic plasticity markers induced by 33i is in line with previous studies suggesting that SIRT2 inhibition could increase glutamate function (Erburu *et al.* 2017). In addition, 33i hyperacetylated α-tubulin in cell cultures (Suzuki *et al.* 2012; Mangas-Sanjuan *et al.* 2015; Rumpf *et al.* 2015). Moreover, we observed that 33i treatment increased p-HDAC5 suggesting perhaps that HDAC5 could be also an enzyme

downstream cytoplasmic SIRT2 function. Altogether, SIRT2 downregulation could be another mechanism by which antidepressants stimulate neuroplasticity.

In summary, this study supports the validity of SH-SY5Y cultures as an *in vitro* cellular model for studying epigenetic changes linked to synaptic plasticity induced by antidepressants as well as the effect of selective HDAC inhibitors. Specifically we present here two epigenetic mechanisms by which antidepressants might enhance neuronal plasticity and presumably, antidepressant action. Firstly, antidepresssant that elevate noradrenaline levels, induce nucleocytoplasmic export of HDAC5. Secondly, monoaminergic antidepressants downregulate SIRT2. Thus, it is highlighted the therapeutic potential of HDAC5 and SIRT2 as epigenetic targets for major depression.

1.3. Antidepressants increase histone acetylation and synaptic plasticity markers

Here, we studied in SH-SY5Y cells the antidepressant regulation of AcH3, AcH4 as well as BDNF and VGLUT1 as good antidepressant state markers. Interestingly, all these proteins were upregulated by incubation for 24 hours of SH-SY5Y cell cultures with imipramine, fluoxetine and reboxetine. These effects might be directly due to enhanced levels of monoamines, including in the case of imipramine, given that the antimuscarinic drug scopolamine showed no changes.

It is noteworthy to mention that antidepressant incubation of cells for only 2 h also increased histone acetylation. Yet, the fact that neither VGLUT1 nor BDNF levels were changed, suggest that histone acetylation precedes in time the synthesis of plasticity markers. According to these results, other authors have also reported that BDNF is upregulated following longer incubation times (Donnici *et al.* 2008).

Comparatively, repeated antidepressant treatment upregulated AcH3, AcH4 *in vivo* in the PFC of mice as previously reported (Erburu *et al.* 2015a) as well as BDNF and VGLUT1. Our experimental design differs from the study of Erburu *et al.* 2015a in that mice were sacrificed 24 h following the last drug injection. Thus, the observed

maintained increase in histone acetylation and synaptic plasticity markers could be neuroadaptive changes associated to antidepressant therapy.

All these studies support the need to study the therapeutic potential of HDAC5 and SIRT2 for depression treatment. In the following section we studied the effect of the selective SIRT2 inhibitor 33i in the VGLUT1+/- depression model. Unfortunately, due to the lack of enough amount of compound for *in vivo* studies, we could not study the effect of the selective HDAC5 inhibitor in this model.

2. SIRT2 inhibition reverses anhedonia in the VGLUT1+/- depression model

We aimed to study the possible antidepressant effect of the specific SIRT2 inhibitor 33i in the VGLUT1+/- depression model. In this research, we showed that the expression levels of the different members of the superfamily of Hdac enzymes are not altered in the VGLUT1+/- model. On the other hand, the SIRT2 inhibitor 33i showed an antianhedonic action in the VGLUT1+/- mouse model. Furthermore, this compound showed no interaction with specific monoaminergic targets such as serotonin or noradrenaline transporters as well as no affinity to the monoaminooxidase enzyme (MAO).

Chromatin remodeling such as changes in histone acetylation has been suggested to play an important role in the pathophysiology of depression. For instance, experimental models based on exposure to stress, have linked upregulation of the histone deacetylases of class I (Hdac1 and Hdac2), class IIa (Hdac4 and Hdac5) and class III (Sirt2) in the prefrontal cortex (PFC) and the hippocampus to depressive-like behaviours (Tsankova *et al*. 2006; Renthal *et al*. 2007; Sarkar *et al*. 2014; Erburu *et al*. 2015a). By contrast, specific inhibitions of these enzymes were reported to have antidepressant-like activity (Schroeder *et al*. 2013; Erburu *et al*. 2017). In keeping with this data, clinical studies have confirmed a positive correlation between the expression of some HDACs and the depressive state of patients (Belzeaux *et al*. 2010; Hobara *et al*. 2010; Watanabe *et al*. 2015). Further, non-specific HDAC inhibitors were proved to have antidepressant effects in patients (Machado-Vieira *et al*. 2011).

Here we have studied the mRNA abundance of the Hdac's superfamily including class I (hdac1, hdac2, hdac3 and hdac8), class II (hdac4, hdac5, hdac6, hdac7, hdac9 and hdac10) and IV (hdac11) as well as the NAD^+-dependent class III (Sirt1-7) in the PFC of VGLUT1+/- mice, as a genetic model of depression (Garcia-Garcia *et al*. 2009). No alterations in the mRNA expression of any of these enzymes were found. Further this model showed no changes in protein expression of HDAC5, SIRT2 or histone acetylation. Thus, the depressive-like behaviour of VGLUT1+/- mice is not linked to variations in Hdac mRNA expression.

Among the different Hdac's, the class III NAD^+-dependent histone deacetylase SIRT2 plays a role in transcriptional repression of transcription factors that participate in synaptic plasticity, cell proliferation, differentiation and cell survival (Eskandarian *et al*. 2013). Previous studies carried out in our laboratory (Erburu *et al*. 2015a, 2017) shows that SIRT2 could contribute to both stress-induced neuronal adaptations as well as to antidepressant action. Specifically, while chronic stress induces SIRT2 expression, this enzyme is downregulated by monoaminergic antidepressants (fluoxetine, reboxetine and imipramine). Moreover, we have also observed that specific inhibition of SIRT2 with the compound 33i, a 3'-phenethyloxy-2-anilinobenzamide analogue (Suzuki *et al*. 2012), induces the expression of the brain derived neurotrophic factor (BDNF) and shows antidepressant-like activity in the chronic mild stress model (Erburu *et al*. 2015a).

In this experiment, we tested the therapeutic value of SIRT2 inhibition in the anhedonic behaviour of VGLUT1+/- mice (Garcia-Garcia *et al*. 2009). Anhedonia, a core symptom of clinical depression is defined as the decreased ability to experience pleasure. In rodents, it has been largely reported that decreased reactivity to rewards parallels anhedonia in humans, and it is mostly studied by analyzing the intake of, or the preference for sweet solutions over drinking water (Willner, 2005). Firstly, we examined the effect of imipramine as a reference antidepressant drug on anhedonic behaviour of VGLUT1+/- mice. Thus, VGLUT1+/- mice showed a decrease in sucrose intake that would be indicative of anhedonia. On the other hand, imipramine increased significantly sucrose intake showing an antianhedonic action from the fourth week until the end of treatment.

Subsequently, the effect of the 33i compound on anhedonic behaviour of VGLUT1+/- mice was tested and interestingly 33i treatment was able to reverse anhedonic behaviour exhibited by VGLUT1+/- mice.

So far, experimental studies linking changes in HDAC's to depressive-like behaviours focus on animal models based on chronic stress (Tsankova *et al*. 2006; Renthal *et al*. 2007; Sarkar *et al*. 2014; Erburu *et al*. 2015b; Zheng *et al*. 2016). However, anhedonia can also be triggered by deficient glutamate transmission (Lally *et al*. 2015), as is the case for the VGLUT1+/- model. We suggest that while anhedonia induced by adverse environmental risk factors could be linked to epigenetic changes, anhedonia induced by this genetic risk factor might be independent. On the other hand, anhedonic behaviour of VGLUT1+/- mice was fully reversed by 33i treatment after two weeks of treatment. Besides, this antidepressant action was maintained in the third week of treatment. Additionally, this compound had no effect in WT mice suggesting that it has the property to reverse anhedonia of "depressed" mice without altering the natural reward reactivity of healthy mice. In agreement with these results, we have previously shown that chronic 33i treatment reverses CMS-induced anhedonia whereas it does not affect the hedonic behaviour of control animals (Erburu *et al*. 2017). On the other hand, the tricyclic antidepressant imipramine, that elevates 5-HT and noradrenaline in the synaptic cleft, showed an antianhedonic action in both VGLUT1+/- and WT mice matching with previous studies showing the ability of noradrenaline to increase reward reactivity (Tong *et al*. 2006).

Former studies have shown that subchronic 33i treatment increases BDNF, serotonin tissue levels and glutamate receptor subunits in the PFC of mice (Erburu *et al*. 2015a, 2017). Interestingly, these effects could be responsible for the antidepressant profile of 33i. Matching with this idea, antidepressant-like effects of monoaminergic antidepressants have been linked to elevation of BDNF and monoamines in the synaptic cleft as well as to activation of glutamate receptors in the PFC (Stasiuk *et al*. 2017). We further studied here whether 33i interacts with specific monoaminergic targets for depression treatment aside from its ability to inhibit SIRT2. Specifically, the compound 33i showed no affinity for the 5-HT transporter or inhibitory activity over [^{3}H]-noradrenaline uptake. Moreover, 33i had no effect on activity MAO enzyme

activity. Thus, these studies suggest that the antidepressant profile of 33i is not linked to a direct interaction with these targets. Yet, we cannot exclude the possibility that the observed effects of 33i on BDNF, serotonin and glutamate receptors could be mediated by other mechanisms independent of SIRT2 inhibition. Chromatin immunoprecipitation studies should be carried out in the future in order to answer this relevant question.

Altogether, these results show that while anhedonia in the VGLUT1+/- model is not linked to variations in Hdac mRNA expression, the selective SIRT2 inhibitor 33i reverses anhedonia in different animal models. It is highlighted the need to further investigate the role of SIRT2 inhibitors as antidepressant agents. Similarly, we are currently studying the role of selective HDAC5 inhibitors as potential antidepressant agents in animal models.

3. Role of VGLUT1 in antidepressant action

In the present study we have used the VGLUT1+/- heterozygous mouse model to further examine the role of prefrontocortical glutamatergic signaling on depressive-like behaviour. Specifically, using the adeno-associated virus (AAV) technology to express the VGLUT1 gene, we aimed to increase VGLUT1 expression in PFC neurons of VGLUT1+/- and WT mice. Firstly, we studied the efficiency of VGLUT1 expression in this area as well as in projecting areas such as glutamatergic descending fibbers going to the dorsal raphe nucleus (DRN). The consequences of this manipulation on depressive behaviour as well as on 5-HT_{1A} function were subsequently investigated.

3.1. VGLUT1 induced expression in the PFC and DRN using AAV-technology

The functionality of the designed plasmid (pAAV-pSyn-VGLUT1-mCherry-minisog-SMD2) was confirmed in rat hippocampal cultures. As expected, VGLUT1^{mCherryminisog} was highly expressed in hippocampal neurons both in the soma and in fibbers which confirms the correct distribution of it. Given that VGLUT1 gene was under the synapsin promoter we can assume that VGLUT1 expression occurs only in neurons.

Subsequently, preliminary experiments were performed to determine the optimal dose of AAV required for imaging VGLUT1^{mCherryminisog} expression in the PFC of WT and VGLUT1+/- mice. Using the highest dilution (1:2), imaging studies revealed VGLUT1^{mCherryminisog} expression in both cell bodies and fibbers in the PFC as well as in fibbers in the DRN. Yet, using lower dilutions (1:4-1:40) of the AAV, no signal at the level of the DRN was detected. Therefore the 1:2 dilution was selected for subsequent studies, as the optimal AAV concentration that allows VGLUT1^{mCherryminisog} expression not only in the site of injection but also in PFC innervating areas. Further, analysis of mCherryminisog or YFP fluorescence in the whole PFC of both genotypes, WT and VGLUT1+/-, revealed no differences in the amount of fluorescence detected (signal area x mean intensity) indicating that reduced VGLUT1 levels in the heterozygous mice (Tordera *et al*. 2007; Garcia-Garcia *et al*. 2009) does not affect to the efficiency of the AAV infection. Given the lack of a good antibody against VGLUT1^{mCherryminisog}, we propose the analysis of amount of fluorescence in the whole area of infection to

estimate the efficiency of expression. Yet, future studies using a specific antibody will allow us to detect whether at this dilution we are able to rescue VGLUT1 levels in the heterozygous mice.

Of the three VGLUT isoforms, VGLUT1 is the most abundant isoform in cortical neurons compared to other areas (Fremeau *et al*. 2001; Hartig *et al*. 2003). Previous studies carried out in our laboratory have confirmed that VGLUT1 heterozygous mice expressed half the amount of transporter in the PFC) compared to WT littermates (Tordera *et al*. 2007; Garcia-Garcia *et al*. 2009). Moreover, reduced VGLUT1 expression was also detected in the DRN (Garcia-Garcia *et al*. 2013). Here, we show a method by which we could increase the expression of VGLUT1 in the PFC and DRN of VGLUT1+/- and WT mice. Importantly, the observation that VGLUT1^{mCherryminisog} is detected in fibbers suggest that VGLUT1 is able to reach synaptic terminals, in where, it should exert its function as a carrier of glutamate into the synaptic vesicles. Moreover, our results agree with previous findings showing that glutamate innervation to the DRN arises in part from the PFC (Artigas *et al*. 1999; Hajós *et al*. 1998; Gonçalves *et al*. 2009; Soiza-Reilly and Commons 2014). In addition, the observed VGLUT1 containing axon terminals in the DRN gives direct evidence of the PFC raphe glutamate pathway and strongly suggest that VGLUT1 is the only isoform responsible for this pathway.

3.2. VGLUT1 induced expression in the PFC rescues depressive-like behaviour in VGLUT1+/- mice

Anhedonia has been largely associated to a dysfunction of the PFC in depressed patients (Keedwell *et al*. 2005) and in rodents (Tordera *et al*. 2007). Previous studies in our laboratory has shown that VGLUT1+/- mice show depressive-like behaviour and increased vulnerability to anhedonia after chronic stress (Garcia-Garcia *et al*. 2009) suggesting that reduced VGLUT1 could be a potential biological risk factor of major depression. In keeping with these studies, VGLUT1+/- mice showed anhedonia measured as a decrease of sucrose intake compared to WT littermates. PFC pAAV-pSyn-VGLUT1^{mCherryminisog} injection increased significantly sucrose intake in both genotypes from the first week suggesting that VGLUT1 expression induces a rapid antianhedonic action. Interestingly this antianhedonic action was maintained up to

week 7 after which, mice were sacrificed. This is the first evidence that link VGLUT1 to antianhedonic behaviour.

The mechanisms by which increased expression of VGLUT1 could lead to anti-anhedonic behaviour should be further explored. VGLUT1 has a key role in regulation of glutamate secretion (Wojcik *et al*. 2004) and this neurotransmitter is linked to mechanisms of neural plasticity. One possible explanation is that VGLUT1 overexpression could enhance glutamate transmission in the PFC leading to an antidepressant action. Matching with this, activation of glutamate receptors in PFC has been linked to antidepressant like effects in patients and rodents (Li *et al*. 2010; Koike *et al*. 2011). Importantly, induced VGLUT1 expression in the PFC of adult VGLUT1+/- mice was sufficient to induce antidepressant action suggesting that early life neuroadaptive changes or other brain regions have little contribution to anhedonia in this model.

Another possible explanation is that, VGLUT1 overexpression could enhance glutamate release from fibbers located in the raphe nuclei and facilitate serotonin release in the forebrain inducing an antidepressant effect. In keeping with this, glutamatergic descending pathways from the PFC are suggested to modulate 5-HT activity in the DRN (Celada *et al.* 2001; Hajós *et al.* 1998; Varga 2001; Tao and Auerbach 2003). Interestingly, the observed inhibition of marble burying behaviour in PFC AAV-pSyn-VGLUT1^{mCherryminisog} injected mice would agree with this idea, since 5-HT active compounds, specially selective serotonin reuptake inhibitors (SSRIs) (Hirano *et al*. 2005; Elizalde *et al*. 2010) also inhibit marble burying behaviour. Further, the sensitivity of the inhibitory 5-HT_{1A} autoreceptors of 5-HT cell bodies having a key role on 5-HT release and mood (Blier and de Montigny 1983; Verge *et al*. 1985; Sprouse and Aghajanian 1987; Richardson-Jones *et al.* 2010) could be also modulated by VGLUT1. In keeping with this, 5-HT_{1A} desensitization observed in the VGLUT1+/- mice (Garcia-Garcia *et al*. 2013) could be a compensatory action for the low glutamate release in the DRN from fibbers descending from the PFC. Conversely, increasing VGLUT1 expression in the PFC of these mice could lead to a recovery of the long-loop excitatory modulation of 5-HT activity and consequently of the 5-HT_{1A} autoreceptor function. In order to prove this hypothesis, we initiated here studies directed to stablish a link between VGLUT1

expression and 5-HT_{1A} autoreceptor sensitivity. While no chanches in 5-HT_{1A} mRNA was found along development, systemic administration of the 5-HT_{1A} agonist 8-OH-DPAT induced a lower hypothemic response in VGLUT1+/- compared to WT littermates which would be indicative of 5-HT_{1A} autoreceptor desensitization (Bill *et al.* 1991), as previously described (Garcia-Garcia *et al.* 2013). Interestingly, injection of AAV-pSyn-VGLUT1^{mCherryminisog} in the PFC of VGLUT1+/- mice induced a hypothermic response to 8-OH-DPAT similar to that observed in WT mice. These results suggest that increased VGLUT1 rescues the sensitivity of 5-HT_{1A} autoreceptors in the DRN. Yet further electrophysioloical and GTP-γ-binding biochemical studies should be carried out to confirm for the first time whether VGLUT1 levels modulate 5-HT_{1A} autoreceptor function in the DRN.

Finally, future studies should explore whether VGLUT1 overexpression in the PFC could also modulate other glutamatergic descending pathways to brain areas involved in the processing of other behaviours. For instance, the mild levels of anxiety observed in PFC AAV-pSyn-VGLUT1^{mCherryminisog} injected mice could be associated to a stimulation of the PFC-amygdala pathway.

Altogether, this study shows for the first time that the stereotaxic injection of an adeno-associated viral vector encoding vesicular glutamate transporter 1 into the PFC results in sustained expression of VGLUT1 at levels sufficient to correct the depressive phenotype in VGLUT1 heterozygous mice (VGLUT1+/-) and to induce antidepressant action in WT.

CONCLUSIONS

1. The antidepressants imipramine and reboxetine increased the phosphorylated form of the histone deacetylase 5 (p-HDAC5) both *in vitro* and *in vivo* in the prefrontal cortex (PFC) of mice, and promoted cytoplasmic export of this enzyme from the nucleus of the cells. Further, specific inhibition of HDAC5 with the compound MC3822 increased histone acetylation and synaptic plasticity markers. These studies suggest that nucleocytoplasmic export of HDAC5 is an epigenetic mechanism by which these antidepressants stimulate neuroplasticity.

2. The antidepressants fluoxetine, reboxetine and imipramine downregulated the histone deacetylase SIRT2 both *in vitro* and *in vivo*. Further, specific inhibition of SIRT2 with the compound 33i increased histone acetylation and synaptic plasticity markers. Thus, SIRT2 downregulation is another epigenetic mechanism by which antidepressants stimulate neuroplasticity.

3. Nucleocytoplasmic export of HDAC5 could be an epigenetic mechanism exclusive of antidepressants able to elevate noradrenaline levels. On the other hand, SIRT2 downregulation could be an epigenetic mechanism of antidepressants that elevate either serotonin or noradrenaline in the synaptic cleft. These are two epigenetic mechanisms by which antidepressants might contribute to increase synaptic plasticity.

4. The selective SIRT2 inhibitor 33i reversed anhedonia in mice heterozygous for the vesicular glutamate transporter 1 (VGLUT1+/-), considered a genetic model of depression. Yet, this model showed no changes in the expression of the selected epigenetic markers (HDAC's, histone acetylation) or in the syaptic plasticity markers. This result evidence that SIRT2 inhibition is able to reverse depressive-like behaviours even when these are not associated to epigenetic alterations.

5. The stereotaxic injection of the designed adeno-associated viral (AAV) vector encoding vesicular glutamate transporter 1 (VGLUT1) into the PFC resulted in sustained expression of VGLUT1^{mCherryminisog} in both cell bodies and fibbers in the PFC as well as in fibbers in the dorsal raphe nucleus (DRN).

6. VGLUT1-induced expression in the PFC of adult mice triggered an antianhedonic action both in VGLUT1+/- and WT mice. In addition, it appears that it rescues the 5-HT_{1A} autoreceptor function in the heterozygous mice. Altogether this is the first evidence linking VGLUT1 to antidepressant action.

7. Taken together, the histone deacetylase enzymes HDAC5 and SIRT2 as well as the synaptic plasticity marker VGLUT1 could be proposed as pharmacological targets involved in antidepressant action. The therapeutic potential of these targets should be further investigated.

1. Los antidepresivos imipramina y reboxetina aumentaron la forma fosforilada de la histona desacetilasa 5 (p-HDAC5) tanto *in vitro* como *in vivo*, en la corteza prefrontal de los ratones. Es decir el tratamiento con estos antidepresivos favorece la traslocación de esta enzima del núcleo al citoplasma de las células. Además, la inhibición específica de HDAC5 con el compuesto MC3822 provocó un aumento de la acetilación de histonas y de los marcadores de plasticidad sináptica. Estos estudios sugieren que la traslocación nucleocitoplasmática de HDAC5 es un mecanismo epigenético mediante el cual estos antidepresivos estimulan la neuroplasticidad.

2. Los antidepresivos fluoxetina, reboxetina e imipramina disminuyeron la expresión de la histona desacetilasa SIRT2 tanto *in vitro* como *in vivo*. Además, la inhibición específica de SIRT2 con el compuesto 33i provocó un aumento de la acetilación de histonas y de los marcadores de plasticidad sináptica. Por lo tanto, la disminución de SIRT2 es otro mecanismo epigenético por el cual los antidepresivos estimulan la neuroplasticidad.

3. La exportación nucleocitoplasmática de HDAC5 podría ser un mecanismo epigenético exclusivo de los antidepresivos cuyo mecanismo de acción eleva los niveles de noradrenalina. Por otro lado, la disminución de SIRT2 podría ser un mecanismo epigenético de los antidepresivos que elevan tanto la serotonina como la noradrenalina en la sinapsis. Estos son dos mecanismos epigenéticos por medio de los cuales los antidepresivos podrían aumentar la plasticidad sináptica.

4. El inhibidor selectivo SIRT2 33i revirtió la anhedonia en ratones heterocigotos para el transportador de glutamato vesicular 1 (VGLUT1+/-), considerado un modelo genético de depresión. Sin embargo, este modelo no mostró cambios en la expresión de los marcadores epigenéticos seleccionados (enzimas histona desacetilasas y acetilación de histonas) ni en los marcadores de la plasticidad sináptica. Este resultado evidencia que la inhibición de SIRT2 es capaz de revertir un fenotipo depresivo también cuando éste no está asociado a alteraciones epigenéticas.

5. La inyección estereotáxica en la corteza prefrontal del virus adenoasociado diseñado en este estudio que codifica el transportador de glutamato vesicular 1 (VGLUT1), dio como resultado un aumento de VGLUT1^{mCherryminisog} tanto en el soma de las neronas, como en fibras en la corteza prefrontal, así como en fibras en el núcleo dorsal del rafe.

6. La expresión inducida de VGLUT1 en la corteza prefrontal de ratones adultos provocó una acción antianhedónica tanto en ratones VGLUT1+/- como en ratones WT. Además, parece que la función del autorreceptor 5-HT_{1A} de los ratones heterocigóticos se normaliza. En conjunto, esta es la primera evidencia que relaciona directamente VGLUT1 con la acción antidepresiva.

7. Recapitulando, podrían proponerse como dianas farmacológicas implicadas en la acción antidepresiva tanto las enzimas histona desacetilasa HDAC5 y SIRT2, como el marcador de plasticidad sináptica VGLUT1. El potencial terapéutico de estas dianas farmacológicas debería investigarse.

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