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TESIS DOCTORAL

INFLUENCIA DEL RECEPTOR 5-HT$_4$ EN LA DEPRESIÓN Y EN EL MECANISMO DE ACCIÓN DE LOS FÁRMACOS ANTIDEPRESIVOS

Josep Amigó Riu

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Directores: Elena Castro y Álvaro Díaz

Mª ELENA CASTRO FERNÁNDEZ, Profesora Titular de la Universidad de Cantabria y **ÁLVARO DÍAZ MARTÍNEZ** Profesor Titular de la Universidad de Cantabria

CERTIFICAN:

Que la Tesis Doctoral titulada "Influencia del receptor 5-HT_4 en la depresión y en el mecanismo de acción de los fármacos antidepresivos", ha sido realizada por Josep Amigó Riu, bajo su dirección en el Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) (UC-CSIC-SODERCAN), con el fin de optar al grado de Doctor.

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Fdo: M. Elena Castro Fernández Álvaro Díaz Martínez

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"*La ciencia es una forma de pensar, mucho más que un cuerpo de conocimientos.*"

Carl Sagan

I. Introduction

1. SEROTONERGIC SYSTEM

Brain serotonergic circuitries interact with other neurotransmitter systems on a multitude of different molecular levels. In humans, as in other mammalian species, serotonin (5-HT) plays a modulatory role in almost every physiological function. 5-HT is the focus of much interest due to its implication in almost every physiological function (eating, reward, thermoregulation, cardiovascular regulation, locomotion, pain, reproduction, sleep-wake cycle, memory, cognition, aggressiveness, response to stressors, emotion, and mood).

Furthermore, dysfunction of the serotonergic systems is thought to be associated with irritable bowel syndrome, restless legs syndrome, sudden infant death syndrome, autism, headache, insomnia, anxiety, depression, anorexia, schizophrenia, Parkinson's disease, and Alzheimer's disease. At the present time, most of the anxiolytic/antidepressant compounds (tricyclic antidepressants and selective serotonin reuptake inhibitors, SSRIs) target the serotonergic systems (reviewed by Charnay and Léger, 2010).

1.1. Neuroanatomy

In 1964, Dahlstrom and Fuxe, using the technique of histofluorescence, observed that the majority of serotonergic soma were found in cell body groups, which had been previously designated by Taber, Brodal, and Walberg as the raphe nuclei.

The soma of these neurons are restricted to discrete clusters or groups of cells located along the mid-line of the brainstem, from where they send axonal projections to nearly every area of the central nervous system. Dahlstrom and Fuxe described nine groups of serotonin-containing cell bodies, which they designated B1 through B9 (Figure 1A). Some serotonergic neuronal cell bodies, however, are found outside the raphe nuclei, and not all of the cell bodies in the raphe nuclei are serotonergic.

Ascending projections from the raphe nuclei to forebrain structures are organized in a topographical manner. The dorsal (B6 and B7) and median (B5, B8 and B9) raphe nuclei give rise to distinct projections to forebrain regions, which can share patterns of innervation. In general, the median raphe projects heavily to the dorsal

hippocampus, septum and hypothalamus, whereas the dorsal raphe heavily innervates the ventral hippocampus, amygdala and striatum. Moreover, raphe neurons send collateral axons to areas of brain that are related in function such as the amygdala and hippocampus, or substantia nigra and caudate putamen.

The other raphe nuclei, B1 to B4, are more caudally situated (mid-pons to caudal medulla) and contain a smaller number of serotonergic cells. These cell body groups give rise to serotonergic axons that project within the brainstem and to the spinal cord (reviewed by Charnay and Léger, 2010; Hensler, 2012).

Therefore, serotonergic fibers innervate several brain regions involved in the major depression disorder (figure 1A). Medial prefrontal cortex is a region involved in the regulation of emotion and assessment of consequence in decision-making. Orbitofrontal cortex is a region involved in the integration of multi-modal stimuli and assessment of stimulus value and reward. Anterior cingulate cortex is a region involved in the emotional processing and autonomic response. Thalamus is a sensory relay and present several connections with other brain structures involved in the emotional regulation. Hippocampus is a region involved in learning and the encoding of memories with the emotional information. Amygdala is a region involved in the evaluation of experience/stimuli with strong emotional value and the acquisition of emotionally related memories. Hypothalamus is a region that links the neuronal and endocrine system to control the hypothalamus-pituitary-adrenal axis (HPA). Nucleus accumbens is a region that plays a crucial role in stimulus-reward processing. (reviewed by Charnay and Léger, 2010; Hensler, 2012).

5-HT$_4$ receptors, the "*leitmotiv*" of this thesis, are strategically located in all these brain areas, granting them a modulating role upon the serotonergic system functioning (figure 1B).

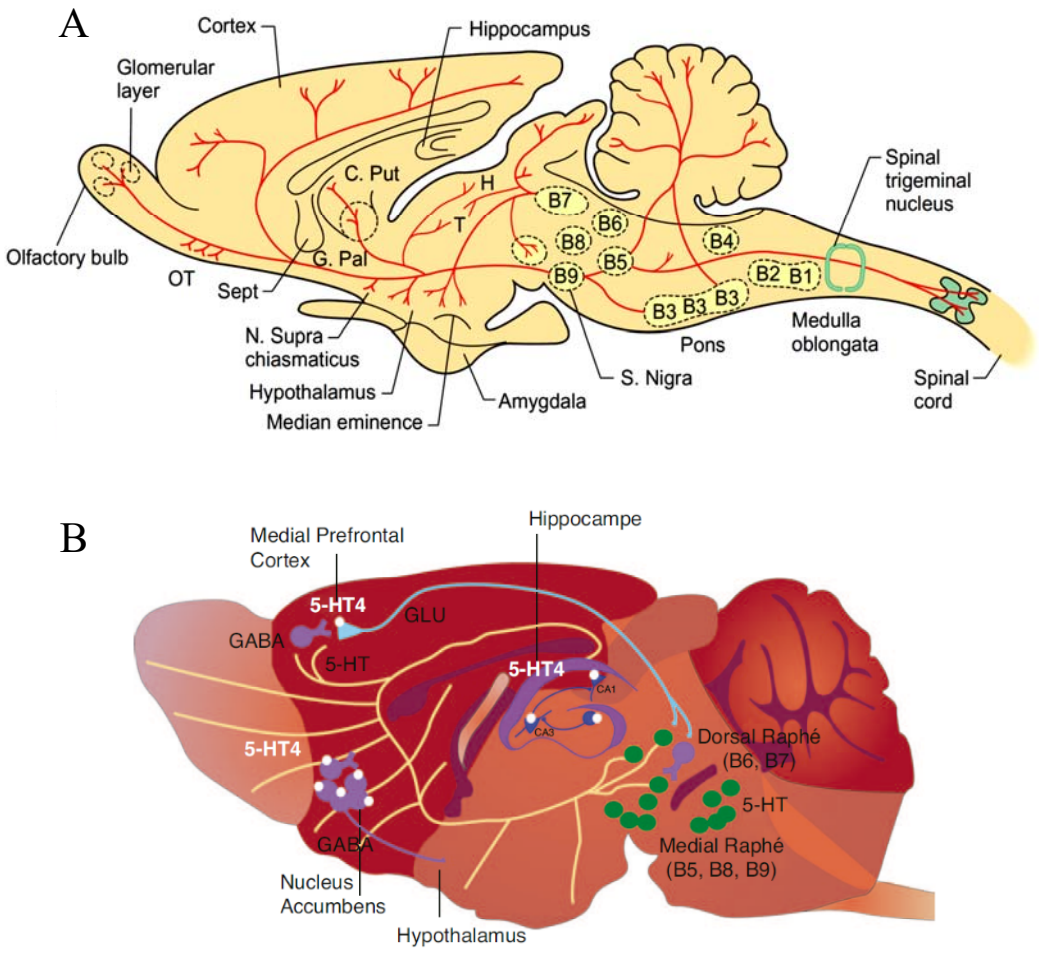


Figure 1. Schematic drawing (**A**) depicting the location of the serotonergic cell body groups in a sagittal section of the rat central nervous system and their major projections. The location of 5-HT_4 receptors in brain regions involved in the depressive disorder is also shown (**B**). OT, olfactory tuberculum; Sept, septum; C. Put, nucleus caudate-putamen; G. Pal, globus pallidus; T, thalamus; H, habenula. A: taken from Hensler, 2012; B: taken from Bockaert *et al.,* 2011.

1.2. Biosynthesis, storage and metabolism

Serotonin is a biogenic monoamine that is synthesized in the serotonergic neurons in the central nervous system and intestinal enterochromaffin by two reactions. Firstly, the essential amino acid L-tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the tryptophan hydroxylase, the rate-limiting enzyme. Secondly, 5-HTP is decarboxylated to synthetize serotonin (5-HT), which is stored in vesicles to avoid its degradation. In the central nervous system, upon neuronal depolarization, serotonin is released into the synaptic cleft. After released, serotonin can bind to its autoreceptors and heteroreceptors, can diffuses into the extracellular space and can be recaptured by the serotonin transporter (SERT). Once inside the neuron, serotonin

is re-stored in vesicles or metabolized in two steps by the monoamine oxidase (MAO), mainly by subtype A, and aldehyde dehydrogenase to become 5-hydroxyindol acetic acid (5-HIAA) (Diaz and Pazos, 2014).

1.3. Serotonin transporter

The main physiological role of the SERT, located in the terminal axons as well as in the cell bodies of the serotonergic neurons, is the clearance of the released 5-HT from the extracellular space, and thus the control of the duration and magnitude of neurotransmission. It has 12 transmembrane domains with the amino and carboxy-terminal groups located in the cytoplasm, with five intracellular and six extracellular loops. Serotonin transporter belong to sodium-symporter family or Na^+/Cl^- dependent transporters. The SERT presents a binding site for those antidepressants known as selective inhibitors of serotonin reuptake (SSRI), thus its blockade will increase the levels of 5-HT in the synaptic cleft and in the vicinity of the serotonergic soma and dendrites. However, the binding of an SSRI to its binding site on the molecule decreases the affinity of the transporter for serotonin (negative allosteric modulation), inhibiting its union. Recently, the crystal structure of the serotonin transporter was resolved (Coleman *et al.,* 2016), finding a main and an allosteric of binding site for citalopram.

Their distribution in the brain have been described employing a range of selective radioligands including [^{3}H] imipramine (Cortés *et al.,* 1988; Kovachich *et al.,* 1988), [^{3}H]paroxetine (Cortés *et al.,* 1988), [^{11}C]DASB (Hipolide *et al.,* 2005) and [^{3}H]citalopram (Varnas *et al.,* 2004). The regional distribution of the SERTs corresponds to areas of the brain that contain serotonergic neuronal bodies and their axonal endings. The higher densities are found in the raphe nuclei, especially in the dorsal raphe nucleus. Medium densities are found in the striatum (caudate, putamen and ventralpallidum), thalamus, and the outermost layers of the entorhinal cortex. Lower densities are found in the rest of cortical structures, the hippocampus (where it presents greater density in the CA3 and dentate gyrus than CA1) and cerebellum (Varnas *et al.,* 2004). On the other hand, the SERT mRNA is mainly located in the raphe nuclei and to a lesser extent in the dorsomedial hypothalamic nucleus (Hoffman *et al.,* 1998).

1.4. Serotonin receptors

Serotonin exerts its actions through at least fourteen receptor subtypes, divided into seven families: 5-HT_1, 5-HT_2, 5-HT_3, 5HT_4, 5-HT_5, 5-HT_6 and 5-HT_7. With the exception of 5-HT_3 receptors that are ligand-activated ion channels, all serotonergic receptors are part of the G protein coupled receptors (GPCRs) superfamily (figure 2) (Hoyer *et al.,* 2002).

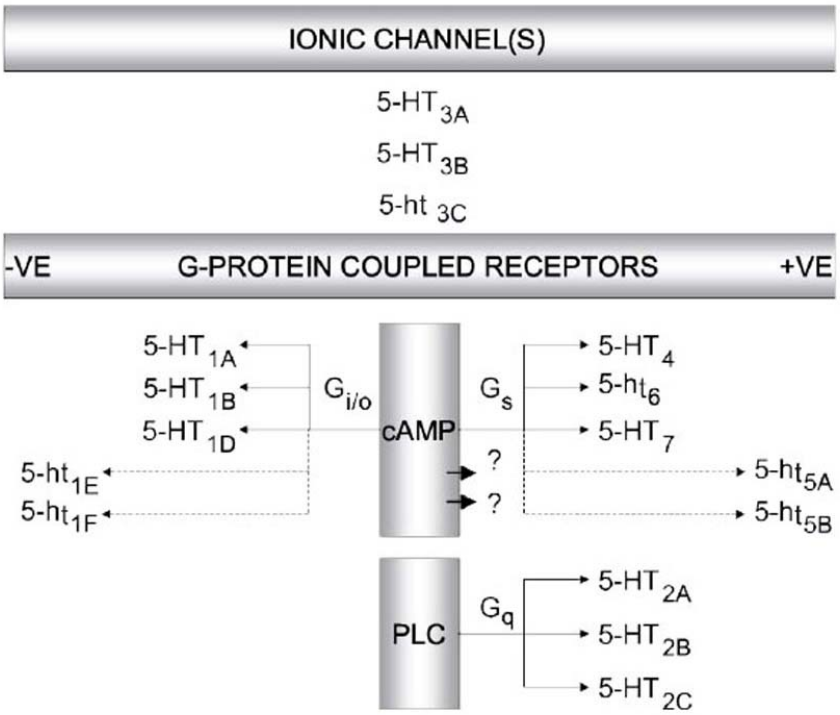


Figure 2. Classification of serotonin receptors and general mechanism transduction. Modified of Hoyer *et al.,* 2002.

Here below we will review the main features of 5-HT_{1A} and 5-HT_4 receptors, which will be studied due to their role in depression and the mechanism of action of antidepressant drugs.

2. 5-HT$_{1A}$ RECEPTOR

This receptor subtype belongs to the 5-HT$_1$ receptor family, and is one of the most studied and characterized serotonergic receptor. They are implicated in the pathophysiology and treatment of depression as evidenced in human studies and animal models of depression (see review Kaufman *et al.,* 2016;Wang *et al.,* 2016 for human studies; Hensler *et al*., 2007; Linge *et al*., 2016; Rainer *et al*., 2012; Schiller *et al*., 2006 for animal studies). The 5-HT$_{1A}$ receptor is a major inhibitory G-protein coupled receptor subtype that exists in two major populations in the nervous system (autoreceptor and heteroreceptor), and functions by coupling to Gi/Go proteins that control numerous intracellular signaling cascades, including inhibition of cAMP formation, inactivation of calcium channels, and activation of potassium channels (Barnes and Sharp 1999).

2.1. Pharmacology

5-HT$_{1A}$ receptor agonists include 8-OH-DPAT that also has affinity for 5-HT$_7$ receptors (Hjorth *et al.,* 1982) and the partial agonist gepirone, ipsapirone and buspirone. The latter is an atypical anxiolytic used in the generalized anxiety disorders (Kumar *et al.,* 2016). Several 5-HT$_{1A}$ antagonists were synthetized such as WAY100,635, WAY100,135, (S)-UH-301 and NAD-299 is the most potent and appears to be the more selective (Barnes and Sharp, 1999).

Pindolol, an β-adrenoceptor antagonist and also partial agonist of 5-HT$_{1A}$ receptor has been evaluated in order to reduce the onset of action of classic antidepressant compounds (Artigas *et al.,* 1996a); however, this therapeutic strategy was not incorporated into the clinical practice due to its pharmacological characteristics. Recently, the FDA approved vilazodone (a dual partial 5-HT$_{1A}$ agonist and SSRI) as an antidepressant drug with fewer side effects and faster onset of action than classic SSRIs (Sahli *et al.,* 2016). Agonists that preferentially target 5-HT$_{1A}$ receptor subpopulations have been synthesized: F15599 that activates 5-HT$_{1A}$ heteroreceptors in the frontal cortex, and F13714 that activates 5-HT$_{1A}$ autoreceptors in the raphe (Garcia-Garcia *et al.,* 2014).

2.2. Functional anatomy

5-HT$_{1A}$ receptors are widely distributed across the brain. Higher densities of this receptor are found in the dorsal and medial raphe nucleus, hippocampus, lateral septum and cingulate and entorhinal cortex (Khawaja, 1995; Pazos and Palacios, 1985). Other cortical and amygdala zones present moderate densities of 5-HT$_{1A}$ receptors. Structures such as other brainstem nuclei, the basal ganglia and the cerebellum show almost undetectable levels of 5-HT$_{1A}$ receptor density (Barnes and Sharp, 1999). 5-HT$_{1A}$ receptors are classified in two subpopulations: the autoreceptors localized somatodendritically in the serotonergic neurons of the raphe nuclei, and the heteroreceptors located postsynaptically in several brain regions innervated by the serotonergic projections.

The activation of somatodendritic 5-HT$_{1A}$ autoreceptors, hyperpolarizes and reduces the firing rate of the serotonergic neurons and, consequently, would decrease serotonin extracellular levels in projection areas (Hjorth and Sharp 1991). Several studies suggest that 5-HT$_{1A}$ receptors in the raphe nuclei are coupled Gαi3 G-protein subunits leading to partial inhibition of adenylyl cyclase (Liu *et al.,* 1999; Valdizán *et al.,* 2010a). Local release of 5-HT in the raphe nuclei diminishes the neuronal firing of 5-HT neurons and produce a negative feedback regulation of transmitter release in different forebrain regions through 5-HT$_{1A}$ receptors among others (see figure 3) (Adell *et al.,* 1991; Artigas *et al.,* 1996b). Consistent with the 5-HT$_{1A}$ autoreceptor role in regulating serotonergic tonus, this autoreceptor could limit the initial increase of extracellular serotonin levels induced by SSRIs (Hjorth and Auebarch, 1994; Hjorth *et al.,* 1996), delaying the clinical antidepressant response (Artigas *et al.,* 1996a,b; Blier and De Montigny 1983; Gardier *et al.,* 1996). This effect disappeared gradually due to several adaptations of the serotonergic system, mainly the desensitization of 5-HT$_{1A}$ autoreceptors in the raphe nuclei (Dawson *et al.,* 2000), allowing the firing rate of serotonergic neurons to recover (Blier and De Montigny 1983).

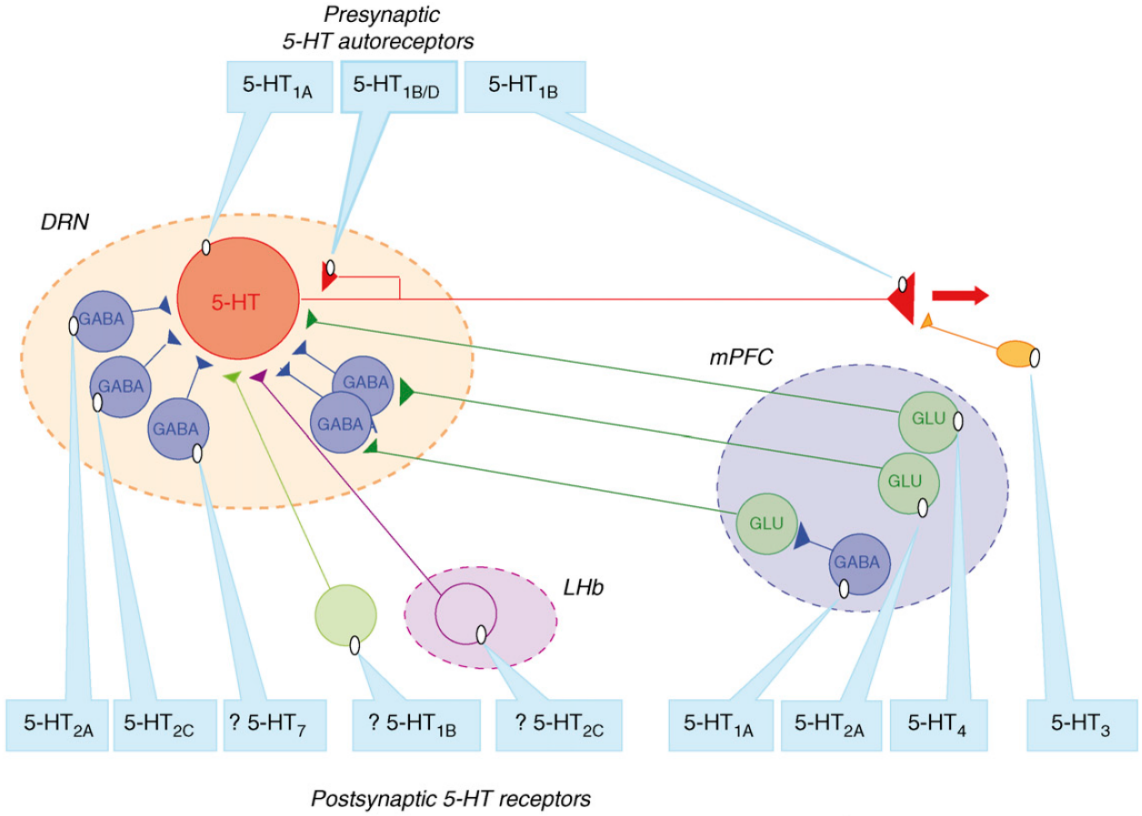


Figure 3. Scheme summarizing the location of serotonin autoreceptors and heteroreceptors involved in controlling the function of serotonin neurons. The activation of both 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptors reduce the cell firing and the release of serotonin in the synaptic cleft. The serotonin heteroreceptors located in the medial prefrontal cortex could also modulate the serotonin neuron activity, though the precise mechanisms by which these heteroreceptors regulate the serotonergic activity are not totally known. For instance, the activation of 5-HT$_4$ and 5-HT$_{2A}$ receptors localized in the glutamatergic neuron of the medial prefrontal cortex could increase the serotonergic neurons firing and the release of 5-HT. By contrast, the activation of 5-HT$_{1A}$ localized in the gabaergic interneurons of the medial prefrontal cortex could reduce the serotonergic neurons firing and the release of serotonin in the synaptic cleft. Taken from Sharp *et al.*, 2007.

In addition to acting as an autoreceptor, 5-HT$_{1A}$ is also a postsynaptic heteroreceptor that mediates responses to released 5-HT in several areas of the brain. These heteroreceptors are mainly located on GABAergic interneurons and pyramidal neurons (Garcia-Garcia *et al.*, 2014) in the prefrontal cortex, hippocampus and amygdala, which are brain regions implicated in the regulation of mood and anxiety. 5-HT$_{1A}$ heteroreceptors are coupled mainly to Gαo subunits in the hippocampus and equally to Gαo and Gαi3 in cerebral cortex (Mannoury la Cour *et al.*, 2001) and the activation of these heteroreceptors induces a hyperpolarizing response (Barnes and Sharp, 2009). Moreover, as shown in figure 3, 5-HT$_{1A}$ heteroreceptors in the mPFC-

raphe pathway could regulate serotonergic neurotransmission (Celada *et al.,* 2001; Hajos *et al.,* 1999).

2.3. Role of 5-HT$_{1A}$ receptors in depression and antidepressant effects

Several studies have investigated the involvement of 5-HT$_{1A}$ receptors in the genesis of the major depressive disorder and the mechanism of action of the antidepressant drugs. Dysregulation of 5-HT$_{1A}$ receptors have been reported in patients suffering from depression, although the *postmorten* and brain imaging studies, which have investigated the 5-HT$_{1A}$ receptor levels in depressed patient, have yielded mixed results. For instance, increases in 5-HT$_{1A}$ autoreceptor density in the midbrain have been reported in depressed suicide victims (Stockmeier *et al.,* 1998). However, Boldrini *et al.,* (2008) demonstrated an increase of 5-HT$_{1A}$ density in the rostral but a decrease in the caudal dorsal raphe nuclei in the brain of depressed patients, suggesting that a complex pattern of 5-HT$_{1A}$ autoreceptor density abnormalities exists in depressed patients. Furthermore, in PET studies a reduction in 5-HT$_{1A}$ autoreceptor and heteroreceptor binding have also been reported in depressed subjects (Bhagwagar *et al.,* 2004; Drevets *et al.,* 2000 and 2007; Meltzer *et al.,* 2004; Sargent *et al.,* 2000)

Despite the discrepancies observed, 5-HT$_{1A}$ receptor is one of the molecular target of several antidepressant and anxiolytic drugs used in the clinical practice. For instance, the anxiolytic properties of buspirone and tandospirone and the antidepressant effects of vilazodone could be due in part to their 5-HT$_{1A}$ receptor partial agonist activity (Sahli *et al.,* 2016; Lacivita *et al.,* 2008; Wang *et al.,* 2016b). Other drugs, such as flesinoxan or flibanserin presented antidepressant efficacy in clinical trials and exhibit high agonist efficacy at 5-HT$_{1A}$ receptors (Pitchot *et al.,* 2005). The co-administration of SSRIs and pindolol accelerates antidepressant response (Artigas *et al.,* 1996a; Portella *et al.,* 2011). All this research supports the importance of 5-HT$_{1A}$ receptors as a therapeutic target in clinical practice.

Pharmacological studies in animals showed that 5-HT$_{1A}$ receptor partial agonists such as buspirone exert antidepressant and anxiolytic effects in animal studies (Detke *et al.,* 1995a, Lucki 1991) and that the behavioural effects of the 5-HT$_{1A}$ agonist 8-OH-

DPAT are absent in 5-HT$_{1A}$ KO (Santarelli *et al.,* 2003). Besides, buspirone and WAY100,635 relieve depression symptoms in the CUMS model (Przegalifiski *et al.,* 1995) and 8-OH-DPAT is effective in the learned helplessness paradigm (Zazpe *et al.,* 2007).

In animal models of depression, alterations in 5-HT$_{1A}$ receptor expression or functionality have been reported. For instance, following maternal deprivation, a reduction of 5-HT$_{1A}$ heteroreceptors and autoreceptors density was found, which was reversed by fluoxetine (Leventopoulos *et al.,* 2009). In the social defeat model, a reduction in the expression of 5-HT$_{1A}$ in the prefrontal cortex was observed (Kieran *et al.,* 2010). In the chronic unpredictable stress, a desensitization of 5-HT$_{1A}$ autoreceptors was found (Bambico *et al.,* 2009). In addition, chronic corticosterone treatment also induces a desensitization of 5-HT$_{1A}$ autoreceptors (Rainer *et al.,* 2012).

Three different lines of 5-HT$_{1A}$ receptor KO mice were generated (Heisler *et al.,* 1998; Parks *et al.,* 1998; Ramboz *et al.,* 1998) and all of them exhibited an anxiety-like phenotype although they did not present a depressive-like phenotype and alterations of 5-HT in projections areas (Bortolozzi *et al.,* 2004; Guilloux *et al.,* 2006; Knobelman *et al.,* 2001). In contrast to the behavioural changes observed in 5-HT$_{1A}$ receptor KO, a transgenic line overexpressing the 5-HT$_{1A}$ receptors in all the brain had reduced anxiety-like behaviour (Kusserow *et al.,* 2004). However, a recent study of our group demonstrates that the overexpression of 5-HT$_{1A}$ receptors in the hippocampus and cortical areas exhibits a high anxious phenotype and an impaired stress coping behaviour (Pilar-Cuéllar *et al.,* 2017). Several conditional 5-HT$_{1A}$ receptor KO mice have been generated in order to study the function of heteroreceptors and autoreceptors in depression and anxiety. For instance, deletion of 5-HT$_{1A}$ autoreceptors throughout life increases anxiety in adult mice and mice lacking 5-HT$_{1A}$ heteroreceptors present an increased behavioural despair in adulthood (Richardson-Jones *et al.,* 2011). In addition, mice with higher or lower autoreceptor levels were generated: mice with higher levels of 5-HT$_{1A}$ autoreceptors showed blunted response to acute stress and increased behavioural despair, whereas both mice showed similar anxiety and levels of serotonin in projection areas (Richardson-Jones *et al.,* 2010). In addition, the loss of forebrain 5-HT$_{1A}$ receptors in the whole life increases the depressive-like behaviour and the knockdown of 5-HT$_{1A}$ receptor of

prefrontal cortex or hippocampus also induces a depressive-like phenotype (Garcia-Garcia *et al.,* 2016a).

Regarding the relevance of 5-HT_{1A} receptors in the mechanism of action of the antidepressant drugs, studies in 5-HT_{1A} KO mice demonstrated that 5-HT_{1A} receptors are necessary for the antidepressant response of SSRIs but not tricyclic antidepressants (Mayorga *et al* 2001; Santarelli *et al.,* 2003). In addition, mice with high expression of 5-HT_{1A} autoreceptors are resistant to the behavioural effects of fluoxetine (Garcia-Garcia *et al.,* 2016b; Richardson-Jones *et al.,* 2010)

Interestingly, the deletion of 5-HT_{1A} receptors on the mature dentate gyrus abolished the fluoxetine antidepressant response in mice, and the expression of 5-HT_{1A} receptors on the granule cells of the dentate gyrus is sufficient to mediate the antidepressant response of fluoxetine (Samuels *et al.,* 2015). Furthermore, the modulation of the 5-HT_{1A} receptor functionality by several antidepressant drugs was extensively studied in *naïve* rodents. A desensitization of 5-HT_{1A} autoreceptors has been found after chronic treatment with SSRIs (Castro *et al.,* 2003a; Rossi *et al.,* 2008) and venlafaxine (Rossi *et al.,* 2006), although this desensitization is produced by different mechanisms depending on the drug studied. Unlike SSRIs and venlafaxine, chronic administration of some tricyclic antidepressants (i.e. desipramine, imipramine) (Blier and de Montigny, 1980) or agomelatine (Hanoun *et al.,* 2004) do not result in the desensitization of the somatodendritic 5-HT_{1A} receptor.

In conclusion, despite all these divergent preclinical, neuroimaging and genetic findings on the relationship between 5-HT_{1A} receptors and the pathophysiology of major depressive disorders, this receptor seems to be crucial in mechanism of action and pharmacological effects of antidepressant drugs.

3. 5-HT_4 RECEPTORS

3.1. Molecular structure

More than 20 years ago, the rat 5-HT_4 receptor cDNA was cloned (Gerald *et al.,* 1995). Two cDNAs were isolated, 5-HT_{4S} and 5-HT_{4L}, nowadays are known as 5-

HT_{4A} and 5-HT_{4B}. After then, several 5-HT_4 receptor cDNAs have been isolated in rat, mouse and human tissues: 4 splice variants in rats (Ray *et al.,* 2009), 10 in humans (Bockaert *et al.,* 2006) and 4 in mice (see Bockaert *et al.,* 2004). The sequences of the different 5-HT_4 receptor isoforms are identical throughout the first 358 amino acid residues, but then diverge, which results in differential G protein coupling (Bockaert *et al.,* 2004). More recently, four 5-HT_4 receptors isoforms in mouse were reported, though they remain to be biologically characterized (Azim *et al.,* 2012). The human 5-HT_4 receptor gene is one of the most complex G-protein-coupled receptor genes (700kb) and presented 38 exons (Hiroi *et al.,* 2001). In mouse, 5-HT_4 receptor gene originally presented 6 exons, however, in 2012, 5 more exons were discovered (Azim *et al.,* 2012). The exact functional roles of the distinct isoforms are unknown. However, the distinct isoforms present some differences in their signalling properties (Bockaert *et al.,* 2004), although their pharmacological properties are similar (Bender *et al.,* 2000).

3.2. Transduction mechanism

Since the discovery of the 5-HT_4 receptor due to its ability to activate the adenylate cyclase, the Gs/cAMP/PKA is the most studied signalling pathway coupled to this receptor (Dumuis *et al.,* 1988; Bockaert *et al.,* 1990). The increase of cAMP induced by the stimulation of 5-HT_4 receptors activates PKA, inducing a long-lasting inhibition of voltage-gated K^+ channels, leading to increased neuronal excitability (Ansanay *et al.,* 1995; Bockaert *et al.,* 2006). In addition, 5-HT_4 receptor activation through cAMP-PKA pathway can activate or inhibit GABAergic synaptic transmission in pyramidal neurons of the prefrontal cortex and hippocampus (Bianchi *et al.*, 2002; Cai *et al.,* 2002).

5-HT_4 receptors also activates the ERK signalling pathway (Barthet *et al.,* 2007; Bockaert *et al.,* 2011; Restivo *et al.,* 2008). This 5-HT_4-mediated ERK stimulation may have a functional relevance for long-term potentiation (LTP) (Huang and Kandel, 2007). In addition, the group of Paul Greengard discovered the interaction of 5-HT_4 receptors with p11; this interaction increases the membrane expression of 5-HT_4 receptors and, subsequently, enhances the signal transduction (Warner-Schmidt *et al.,* 2009).

3.3. Brain regional distribution

The brain distribution and pharmacological characterization of the 5-HT_4 receptor is already known, using radioligand binding techniques and ligands such as [^{3}H]GR113808 (Grossman *et al.,* 1993). The distribution observed among different species is quite similar (Waeber *et al.,* 1993, 1994): a high density is observed in the basal ganglia, especially in the nucleus accumbens, caudate-putamen, globus pallidus, and substantia nigra, and minor densities are observed in other brain areas such as the hippocampus, amygdala, cortical areas, thalamus or hypothalamus. This autoradiographic distribution of 5-HT_4 receptors is in line with findings in neuroimaging and *in situ* hibrydization studies (Kornum *et al.,* 2009; Vilaró *et al.,* 2005).

In cortico-limbic areas, 5-HT_4 receptors are expressed in glutamatergic pyramidal neurons in the medial prefrontal cortex and the hippocampus (Roychowdhury *et al.,* 1994; Tanaka *et al.,* 2012; Vilaro *et al.,* 2005). They are also expressed in cholinergic neurons in the cortex, hippocampus, and amygdala (Waeber *et al.,* 1994), in GABAergic striatal spiny efferent neurons (Cai *et al.,* 2002; Compan *et al.,* 1996) and in efferent neurons of the nucleus accumbens that project to the lateral hypothalamus (Jean *et al.,* 2012) (figure 1B).

3.4. Pharmacology

There are several ligands of 5-HT_4 receptors (Bockaert *et al.,* 2004), the most relevant will be mentioned. Among 5-HT_4 agonists, the first generation of benzamides, such as the zacopride, renzapride and metoclopramide are also 5-HT_3 receptor antagonists. Other benzamides are potent 5-HT_4 receptor agonists (cisapride, SB205149, SC53116) with little affinity for 5-HT_3 receptors. The substitution of the amide by an ester led to benzoate derivates with greater affinity than other classes of benzamides for 5-HT_4 receptors and little affinity for 5-HT_3 receptors. ML10302 was the first potent and selective 5-HT_4 partial agonist, though easily hydrolysed that limited its utility. In this class, it was also synthesized SL65.0155 (Mosser *et al.,* 2002), a potent and partial 5-HT_4 receptor agonist. The fourth class of compounds are benzimidazolones such as BIMU-1 and BIMU-8 that are potent 5-HT_4 agonists that

cross the blood brain barrier. RS67,333 is the compound used in the thesis, which has an increased blood brain barrier penetration. This compound is a 5-HT_4 partial agonist with high affinity for 5-HT_4 receptors. However, it also binds with high efficacy to σ_1 and σ_2 receptors (Eglen *et al.,* 1995a). Another interesting partial agonist is the pyridine carboxamide VRX-03011 or NTC-92 developed for the treatment of Alzeihmer´s disease (Ahmad and Nirogi, 2011).

Among the antagonists, GR113808 was the first compound with high affinity for 5-HT_4 receptors and low affinity for 5-HT_3 receptors. In addition, it was the first commercially available radioligand ([^{3}H]GR113808) to label 5-HT_4 receptors. GR125487, an ester analogue of GR113808, is a more potent antagonist with higher bioavailability (Eglen *et al.,* 1995b). Other classes of antagonists include benzoates such as SDZ205557, benzoate dioxanes such as SB204070 (with high affinity but low bioavailability) and SB207710 (which has also been used as radioligand to study the cerebral distribution of the receptor, [^{125}I]SB207710), benzimidazolones (DAU6285) and aryl ketones. Furthermore, some inverse agonists were synthetized such as RO116-0086 and RO116-1148 (Joubert *et al.,* 2002).

3.5. Role of 5-HT_4 receptors in depression and the mechanism of action of antidepressant drugs

Some clinical studies have investigated if the depressive pathology was associated with alteration in the levels or functionality of 5-HT_4 receptors across the brain. Studies in *postmortem* brain samples from depressed suicide victims showed a greater density and functionality of 5-HT_4 receptors in cortical and striatal areas (Rosel *et al.,* 2004). By contrast, *in vivo* PET imaging studies in humans demonstrated that a reduction in 5-HT_4 receptors binding in the striatum is associated with a high risk to suffer from major depression (Madsen *et al.,* 2014). In addition, a moderate reduction in the concentration of 5-HT_4 receptors in both the striatum and amygdala was described in healthy subjects treated with fluoxetine for three weeks (Haahr *et al.,* 2014). Furthermore, the functioning of the HPA axis was negatively associated with the 5-HT_4 receptors binding in several cortical and striatal regions (Jacobsen *et al.,* 2016). From the preclinical approach, two different animal models of depression, olfactory bulbectomised (OBX) and glucocorticoid heterozygous receptor mice,

showed an increase in the expression of 5-HT$_4$ receptor in the ventral hippocampus or striatum, respectively (Licht *et al.,* 2010a). In contrast, a down-regulation of 5-HT$_4$ receptors in the ventral and dorsal hippocampus was reported in the Flinders-sensitive line rat model of depression (Licht *et al.,* 2009).

The 5-HT$_4$ receptors are implicated in the mechanism of action of antidepressants and ligands of this receptor could exert antidepressant effects (Lucas *et al.,* 2007; Vidal *et al.,* 2014). We have previously reported a down-regulation of 5-HT$_4$ receptors in the striatum and hippocampus of rats chronically treated with fluoxetine (Vidal *et al.,* 2009) and venlafaxine (Vidal *et al.,* 2010). A recent study further described that the activation of 5-HT$_4$ receptors may partly mediate some antidepressant and anxiolytic actions of fluoxetine in predictive behavioural paradigms [tail suspension test (TST) for depression and open-field/elevated plus maze tests for anxiety (Mendez-David *et al.,* 2014)] in a model of anxiety/depression based on chronic elevation of glucocorticoids. In this context, it is noteworthy to mention that some of the neurogenic actions induced by SSRIs involve the 5-HT$_4$ receptors (Imoto *et al.,* 2015). However, in *naïve* rats, the decreased immobility in the forced swim test (FST) induced by acute fluoxetine administration is not affected by co-administration of the 5-HT$_4$ receptor antagonist SB 204070A (Cryan and Lucki 2000). In addition, the anxiogenic effects of the drug in the open field and light/dark box was absent in 5-HT$_4$ KO mice, but this mice retained the behavioural effects of fluoxetine in the forced swimming test and tail suspension test (Kobayashi *et al.,* 2011). Therefore, taken together, these findings suggest that activation of 5-HT$_4$ receptors mediates, in part, the antidepressant-like effects of SSRIs.

At a preclinical level, there are not many studies in animal models of depression since most of the behavioural effects of 5-HT$_4$ agonist were evaluated in naive animals. Short-term treatment with a 5-HT$_4$ agonist in rodents and long-term administration of SSRIs induced similar antidepressant/anxiolytic actions (Lucas *et al.,* 2007; Pascual-Brazo *et al.;* 2012; Tamburella *et al.,* 2009; Vidal *et al.,* 2014). Furthermore, the 5-HT$_4$ receptor activation could mediate neurogenesis in the hippocampus of adult rodents (Lucas *et al.,* 2007; Pascual-Brazo *et al.,* 2012). However, there are discrepant evidences since some studies found an anxiolytic effect of the 5-HT$_4$

receptor antagonists SB 204070, GR 113808 and SB 207266A in the elevated plus maze in rats (Kennett *et al.,* 1997; Silvestre *et al.,* 1996).

Despite these evidences about the implication of 5-HT_4 receptors in depression and in the effects of antidepressants, few studies have investigated the behavioural, neurochemical and/or molecular consequences of the genetic ablation of 5-HT_4 receptors. 5-HT_4 KO mice exhibits a reduced firing (-50%) of the DRN 5-HT neurons and serotonin levels in the raphe nuclei, reduced density of the 5-HT_{1A} receptors in the DRN and hippocampus and increased levels of the 5-HT transporter (SERT) (Conductier *et al.,* 2006). Behavioural studies have shown that these mice display abnormal feeding, locomotor and anxiety-like behaviour in response to stress and novelty, seizure susceptibility and long-term memory deficits (Compan *et al.,* 2004; Jean *et al.* 2007; Jean *et al.,* 2012; Segu *et al.,* 2010). However, whether the 5-HT_4 KO mice display specific anxiety- and depression-like behaviours in different contextual situations (e.g. novelty suppressed feeding paradigm as a conflict-based test, sucrose intake as anhedonic test, and chronic depression/anxiety models) remains to be fully explored. Similarly, little is known about possible adaptive changes in brain neuroplasticity and neurogenesis in the absence of 5-HT_4 receptors, despite some pharmacological evidences (Imoto *et al.,* 2015; Pascual-Brazo *et al.,* 2012). In this context, a work with 5-HT_4 KO demonstrated that 5-HT_4 receptor activation could play a role in granule cells dematuration induced by chronic antidepressant treatment (Kobayashi *et al.,* 2010).

4. DEPRESSION

4.1. General features

Major depression disease is a chronic, recurrent and multifactorial mental disorder that puts the patient's life at risk and presents neuroendocrine, physiological, and behavioural features together with the psychological symptoms. It is one of the most prevalent major neuropsychiatric diseases in the developed countries, where the lifetime prevalence could be as high as 20% (Kessler and Bromet, 2013). It is estimated to affect around 350 million people and often starts at a young age, thus, reducing people's capacities. For these reasons, depression is the first cause of

disability worldwide in terms of total years lost due to incapacity (WHO, 2012). Furthermore, depression can lead to suicide and more than half of suicide victims presented a depression diagnostic (Rihmer, 2007).

The diagnosis of the disease, based on a highly heterogeneous set of symptoms, is difficult and favours the underdiagnoses. In Diagnostic and Statistical Manual of Mental Disorders (DSM-V) several modifications have been included that may influence how the diagnosis is used in both clinical and research environments. According to DSM-V a diagnosis of major depression is made when a certain number of the below stated symptoms are reported for longer than two weeks, and when this symptoms disturb normal, social and occupational performances of the patient. The diagnostic criteria of DSM-V for major depressive disorder are the following:

1. Depressed mood (such as sad, empty or hopeless).
2. Loss of interest or pleasure (anhedonia).
3. Change in weight or appetite.
4. Insomnia or hypersomnia.
5. Psychomotor retardation or agitation (observed).
6. Loss of energy or fatigue.
7. Worthlessness or guilt.
8. Impaired concentration or indecisiveness.
9. Thoughts of death or suicidal ideation or attempt.

Moreover, depressive patients could present comorbidity with several others mental disorders such as the generalized anxiety disorder, post-traumatic stress disorder, etc. Hence, depression presents a highly variable set of symptoms that it hinders the discovery of biomarkers. For this reason, a dimensional view of the mental disorders could favours the finding of new reliable biomarkers (Venkatasubramanian and Keshavan, 2016).

4.2. Pathophysiology of depression

The pathophysiology of this heterogeneous disease, as other mood disorders, remains poorly understood. Several hypotheses have been postulated regarding the molecular and neurochemical mechanisms that could underlie the disorder. These hypotheses

are not mutually exclusive and when combined they could provide the "scaffolding" to gain insight in the processes that originates the disease, the mechanism of action of the antidepressant drugs and, subsequently, the basis for developing new treatments.

4.2.1. Genetic component in depression

Depression heritability on twin studies is 40% to 50% (Bierut *et al.,* 1999; Sullivan *et al.,* 2000) reflecting that the genetic component is moderate and environmental factor are equally important. Indeed, in monozygotic twins, sometimes one of the siblings develops the disease and the other does not, therefore, the different behaviour in genetically identical twins could be due to epigenetics differences (Petronis *et al.,* 2003). Nowadays, some studies show how the stress could modify the established genetic mechanisms and alter the neuronal circuitry functioning that confers to individuals an increased susceptibility to develop future mental illnesses (Post, 2016).

Initially, the search for genetic candidates was focused on the cerebral monoaminergic system due to its relevance in depression and its pharmacological treatment. A 44-bp repeat polymorphism in the promoter region of the serotonin transporter gene (SLC6A4) is the most studied and paradigmatic genetic variant of psychiatric genetics. This polymorphism presents two alleles the long (L) and the short (S) variants, the latter being associated with lower functionality and density of the serotonin transporter (Lesch *et al.,* 1996). In a highly cited article, Caspi *et al.,* (2003) demonstrated that subjects with one or more copies of the short allele of the polymorphism presented an increased risk of depression. Additionally, a meta-analysis study found a significant relationship between the long allele and better antidepressant response to SSRI (Serretti *et al.,* 2007).

Additionally, functional polymorphisms in other genes of the serotonergic system have been analysed. In the case of the 5-HT_{1A} receptor, the G/G(-1019) genotype was associated with major depression and suicide (Lemonde *et al.,* 2003). Moreover, case-control association studies of the 5-HT_{2A} receptor and major depressive disorder have yielded mixed results (Anguelova *et al.,* 2003) and a polymorphism in the 5-HT_4 receptor has been associated with the bipolar disorder (Ohtsuki *et al*., 2002). Interestingly, it has been reported an association between some variants in the THP2

gene, the rate-limiting enzyme in brain serotonin synthesis, and major depression (Zill *et al.,* 2004), and some variants of this gene were associated with suicide (Lopez *et al.,* 2007;).

The gene that encodes for the brain-derived neurotrophic factor (BDNF), which is one of the most researched and characterized neurotrophin in the central nervous system, has been studied extensively in relation to depression. The association studies of the single nucleotide polymorphism Val66Met of BDNF with the depressive disorder have reported divergent results (Anttila *et al.,* 2007; Schumacher *et al.,* 2005). However, this genotype is correlated with the hippocampal volume and the increased sensitivity to adverse experiences (Hajek *et al.,* 2012; Hosang *et al.,* 2014), two endophenotypes related to the development of depression.

In the last years, the candidate gene studies have been replaced for the genomic wide association studies (GWAS) where millions of polymorphism are tested at once. The findings of the early GWAS studies of major depression disease (MDD) were poorly reproducible. In recent studies the sample heterogeneity was reduced, increasing the genetic effects detected and favouring the reproducibility of the findings (CONVERGE consortium, 2015). Nevertheless, it would be ideal to perform genome-wide association studies of particular subtypes of depression or some endophenotypes in order to minimize genetic and phenotypic variability (Levinson *et al.,* 2006). On the other hand, mostly of the studies are focused on single nucleotide polymorphism but other DNA variations such as copy number variants, microsatellites, variable number tandem repeats could be also a significant part of the genetic component of the disease.

4.2.2. Monoamine hypothesis

The monoamine hypothesis of depression postulates that depression is directly related to decreased monoaminergic transmission (serotonin, noradrenaline and dopamine) (Coppen, 1967; Schildkraut, 1965). Several studies tried to correlate the depressive state with deficits in the levels of brain monoamines, though the findings were inconclusive. These studies analysed the levels of noradrenaline, 5-HT and their major metabolites, as well as the enzymes involved in their synthesis, in various biological fluids (cerebrospinal fluid, urine and blood) and in *postmortem* cerebral

tissue samples. Only the correlation between low levels of *postmortem* and suicidal behaviour appeared to be confirmed in some studies (Asberg *et al.,* 1976; Risch and Nemeroff, 1992). Moreover, studies in which monoamine depletion was induced by the administration of reserpine, tyrosine hydroxilase inhibitors or the suppression of tryptophan in the diet, concluded that the depressive patients undergoing clinical improvement worsened their symptoms after monoamine depletion (Miller *et al.,* 1996), whereas, non-medicated patients did not get worse (Heninger *et al.,* 1996). In addition, a mismatch was found between the time that the antidepressant required to induce its beneficial effects (usually weeks) and the almost immediate increase in the concentration of monoamines in the synaptic cleft (Bel and Artigas, 1992), challenging the claim that restoration of the brain levels of monoamines is the direct cause of the clinical improvement.

Due to several discordant findings, the monoaminergic hypothesis was reformulated and increased in complexity. In this new context, the depression could be caused to alterations in the synthesis, storage, or release of the monoamines, as well as disruptions in the sensitivity of their receptors or their intracellular signalling mechanisms (Bondy, 2002). Therefore, the research interest shifted from the mere determination of neurotransmitter levels toward the study of the density and functionality of monoaminergic receptors. Among these, noradrenergic subtype β receptors have been frequently associated with depressive disorders. Their density appears to be altered in depression, although studies in the literature are contradictory. It has been observed an increase in the density of β-adrenergic receptors in suicidal subjects compared to healthy controls (Mann *et al.,* 1986; Biegon and Israeli, 1988), though this results was not replicated in the subgroups of suicides with depression disorder (De Paermentier *et al.,* 1990). Besides, chronic treatment with antidepressants desensitizes β-adrenergic receptors (Vetulani *et al.,* 1976; Sulser, 1978) and this time matches with the time required for therapeutic efficiency. These findings suggested that the blockade of monoamines reuptake did not produce therapeutic effects by itself and, thus, some adaptive changes in receptor sensitivity, due to the increased monoamine levels, could account for the antidepressant efficacy. Presynaptic α_2 receptors have also been implicated in depression, since an increase in the density, affinity and efficacy to coupling to G-proteins was observed in brains

of suicidal patients diagnosed with depression (Meana *et al.,* 1992; Callado *et al.,* 1998; Valdizán *et al.,* 2010b). Additionally, the hypersensitization of α_2 receptors localized in the serotonergic neurons of depressed patients could decreased serotonergic transmission in projection areas such as the cortex and hippocampus (Mongeau *et al.,* 1997). These α_2 receptors of the serotonergic fibres are desensitized after chronic antidepressant treatment (Mongeau *et al.,* 1994), favouring the increase of serotonin levels in projection areas, which is observed after antidepressant treatment.

In the case of the serotonergic system, there are several evidences pointing towards a serotonin deficit in some populations of depressed patients. In addition to the studies on the tryptophan and catecholamines depletion in depressed subjects, it was also detected a reduction in the number of binding sites to [^{3}H]imipramine both in the platelets (Briley *et al.,* 1980, Nemeroff *et al.,* 1988~~a~~) and *postmortem* brain samples (Stanley *et al.,* 1982; Perry *et al.,* 1983) of depressed patients. Furthermore, a reduction in the serotonin transporter density was detected in the brain of depressed patients by single-photon emission computed tomography (SPECT) (Malison *et al.,* 1998). This could indicate a deficit in the amine reuptake capacity, or a loss of serotonergic neuron activity in these patients. Moreover, some findings in mice support the view that a serotonin deficiency could leads to a depressive state, since it has been reported that brain serotonin deficiency was associated with psychomotor agitation, disruption of sleep pattern, anxiety and depressive behaviours (Whitney *et al.,* 2016).

An increase in the density of 5-HT$_2$ receptors has also been observed in the frontal cortex and platelets of depressed patients (Arango *et al.,* 1990; Arora and Meltzer, 1989) which could be an adaptive response to the reduced 5-HT synaptic levels observed in depressed patients. However, PET studies with 5-HT$_2$ ligands in depressive subjects provide contradictory results, since increments in the frontal cortex (Meyer *et al.,* 2003), decreases in orbito-cingular cortex (Biver *et al.,* 1997) and hippocampus (Mintun *et al.,* 2004) were reported. Furthermore, there are several stuides in which a decreased brain density of these receptors was found after chronic treatment with antidepressants, both in rodents (Peroutka and Snyder, 1980; Klimek and Papp, 1994) and depressed patients (Yatham *et al.,* 1999). An increase in cortical

5-HT_{2A} receptor have also been described in depressed patients after recovery (Bhagwagar *et al.,* 2006). The 5-HT_{1A} receptor is other serotonin receptor subtype that has been extensively studied regarding the neurobiology of depressive disorders and the mechanism of action of antidepressant drugs. We have already commented about this receptor in a previous section.

4.2.3. Neurotrophic/neuroplastic hypothesis

Neuroplasticity is a set of processes through which external and internal environment of an individual *sculpts* neuronal structure and function. Among neuroplasticity mechanisms, some are trophic processes such as neurogenesis and synaptogenesis, but others are atrophic processes, such as the elimination of inactive neurons and neuronal contacts. In addition, plasticity could be adaptive or maladaptive depending if it is guided by beneficial or adverse experiences (Castrén and Antila, 2017).

There are several evidences that suggest alterations in brain neuroplasticity in the depressive disorder. For instance, brain imaging studies showed a reduction in the volume of the hippocampus and prefrontal cortex of depressed patients (Drevets *et al.,* 2008; Neumeister *et al*., 2005), as well as grey matter abnormalities in the anterior cingulate cortex (Bora *et al.,* 2012), all these brain morphological abnormalities were localized in brain structures implicated in the depressive disorder. *Postmorten* studies in depressed patients have reported a reduction of glial number and dendritic complexity in the hippocampus and frontal cortex, alterations also observed in animal models of depression (Duman and Li, 2012). Moreover, chronic administration of classical antidepressants increases synaptic plasticity at several levels including neurogenesis in the hippocampus, neurotrophic factor expression, and synaptogenesis (Duman *et al.,* 2016). Hence, the neuroplastic/neurotrophic hypothesis proposes that depression results from decreased neurotrophic levels, leading to a reduction in the neuroplasticity mechanisms that induce neuronal atrophy, decreased hippocampal neurogenesis and loss of glia, and that antidepressant treatment reverses these neurotrophic factors deficits, and the alteration in the neural plasticity (Duman and Li, 2012). However, not all the findings fit well with this theory. For instance, neural plasticity in depressed patients might be enhanced in the amygdala, since patients with major depression presented higher volume and increased glucose metabolism in

this brain structure (Kuhn *et al.,* 2014). The neurotrophic/neuroplastic hypothesis does not exclude the participation of the serotonergic system and, as shown in figure 4, there is a crosstalk in the main signalling intracellular pathways linking antidepressant actions to proliferative and/or plasticity changes. The increased 5-HT release in the synaptic cleft, induced by classical antidepressants, could activate GPCR, including 5-HT_{1A} and 5-HT_4 receptors, which, in turn, regulate a plethora of processes implicated in neuroplasticity. Indeed, it is postulated that the antidepressant response is due to adaptive changes related to these neuroplasticity proteins, especially BDNF that seems to be a "key transducer" of the antidepressant effects (Björkholm and Monteggia, 2016).

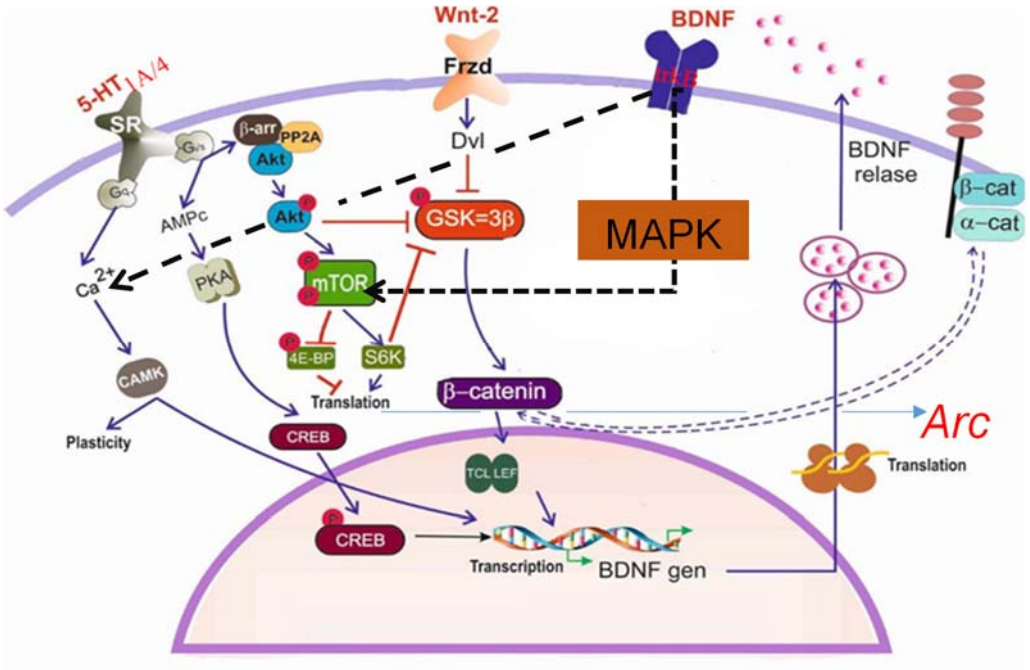


Figure 4. Crosstalk in the main signalling pathways linking antidepressant action to proliferative and/or plasticity changes. Classical antidepressant drugs increase 5-HT release in the synaptic cleft that could activate GPCR, including 5-HT_{1A} and 5-HT_4 receptors. These serotonin receptors regulate intracellular pathways that activate transcription factors such as CREB, whose activation leads to the transcription of several genes of neuroplasticity such as BDNF. The binding of the trophic factor BDNF to its receptor, TrkB, induces a plethora of signalling cascades, highlighting the activation of the PI3K/Akt pathway that leads to the inhibition of GSK-3β and, the subsequent activation of mTOR signalling. mTOR leads to an increased translation of synaptic proteins (PSD95, GLUA1 and *Arc*). The inhibition of GSK-3β also allows the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus, where it plays an important role in synaptic vesicle release. Modified from Pilar-Cuéllar et *al.,* 2014.

BDNF/trkB. The brain-derived neurotrophic factor (BDNF) is one of the most studied neurotrophins. This neurotrophic protein supports neuronal survival and promotes neuroplasticity in the adult brain by activating its cognate receptor, the tyrosine kinase receptor TrkB (Park and Poo, 2013). Clinical studies reported reduced BDNF and TrkB levels in the brain of depressed patients (reviewed in Castrén and Kojima, 2017). Other studies showed a reduction in serum BDNF levels in patients with major depression (see review Molendijk *et al.,* 2014), which were restored to normal values in depressed patients in remission (Shimizu *et al.,* 2003; Sen *et al.,* 2008).

There is also a lot of preclinical research on BDNF and depression after the availability of genetically modified mice for BDNF and TrkB. Heterozygous BDNF KO mice (Korte *et al.,* 1995; MacQueen *et al.,* 2001; Ibarguen-Vargas *et al.,* 2009) presented a complex anxiety- and depression-like behavioural phenotype. A normal anxiety-like behaviour (Chourbaji *et al.,* 2004; Ibarguen-Vargas *et al.,* 2009; Lindholm *et al.,* 2012), but also a more aggressive and anxious phenotype (Lyons *et al.,* 1999) were reported. Heterozygous BDNF KO mice did not exhibit a depression-like response in behavioural despair paradigms though they are resistant to classical antidepressants (Chourbaji *et al.,* 2004; Ibarguen-Vargas *et al.,* 2009; MacQueen *et al.,* 2001). The deletion of BDNF in the hippocampus and cerebral cortex induced hyperactivity in male mice and depressive-like behaviour in female mice (Chan *et al.,* 2006). The local lentiviral *knockdown* of BDNF in the dentate gyrus and the ventral subiculum, but not the CA3, induced anhedonia and depression-like behaviours in rats (Taliaz *et al.,* 2010). Loss of BDNF specifically in the dentate gyrus or CA1 did not affect motor activity, anxiety- and depressive- like behaviour or fear conditioning, whereas, deletion of BDNF in the dentate gyrus prevented the effect of antidepressants drugs in behavioural despair paradigms (Adachi *et al.,* 2008).

Only a few studies have evaluated the behavioural consequences of deleting trkB in mice. Heterozygous trkB mice presented a disruption of circadian rhythm, one of the neurovegetative symptoms of depression (Allen et *al.,* 2005). In addition, mice with TrkB deficiency in the hippocampus and cerebral cortex showed hyperactivity and impulsivity, but did not show anxiety-like and depression-like behaviours (Zörner *et al.,* 2003). Moreover, mice with specific TrkB deletion in adult neuronal progenitors

of the dentate gyrus showed increased anxiety behavior (Bergami *et al.,* 2008), and they did not respond to classical antidepressant treatment (Li *et al.,* 2008).

On the other hand, transgenic mice overexpressing BDNF or trkB have been developed. Mice overexpressing BDNF in glutamatergic neurons of the hippocampus, cortex, and amygdala presented an antidepressant-like behavioural phenotype associated with increased anxiety levels (Govindarajan *et al.,* 2006). However, transgenic mice overexpressing trkB receptor showed reduced anxiety and depressive manifestations (Koponen *et al.,* 2004).

Adaptive changes in BDNF/trkB pathway have been analysed in several animal models of depression. The vast majority reported reduced BDNF levels in the hippocampus and frontal cortex in models such as social isolation, learned helplessness, chronic mild stress and social defeat (Stepanichev *et al.,* 2014), though an increase in the levels of BDNF in the hippocampus was observed in the early maternal deprivation model (Faure *et al.,* 2007). Olfactory bulbectomy is associated with an upregulation of BDNF levels in mice (Hellweg *et al.,* 2007; Linge *et al.,* 2015), but downregulation in rats (Hendriksen *et al.,* 2012; Rodriguez-Gaztelumendi, 2010). Chronic unpredictable mild stress increases (Boulle *et al.,* 2014) o decreases (Jiang *et al.,* 2012, Grønli *et al.,* 2006) hippocampal levels of BDNF. Chronic treatment with antidepressant drugs, electroconvulsive therapy and physical exercise increased BNDF mRNA and protein levels in both humans and rodents (reviewed in Castrén and Kojima, 2017).

To sum up, the increase of BDNF expression induces an antidepressant effect in animal models of depression and control animals. Furthermore, this neurotrophin is necessary for the antidepressant action of classical antidepressants and ketamine (Björkholm Monteggia, 2016). However, it is less clear if a reduction of the levels of BDNF could induce a depressive state due to the divergent results obtained in mice with decreased BDNF expression and in models of depression (Lindholm and Castrén, 2014).

Arc. The activity-regulated-cytoskeleton-associated protein (*Arc*) is an immediate early gene with complex functions that plays a prominent role in the synaptic plasticity events such as the LTP (long term potentiation), LTD (long term

depression) and the remodeling of dendritic spines (Li *et al.,* 2015). As far as we know, only one study investigated the modulation of *Arc* expression in *postmorten* brain of depressed patients, in which a high decrease of *Arc* expression was found in the ventral anterior cingulate cortex (Covington *et al.,* 2010).

In animals, low levels of *Arc* mRNA were reported in the frontal cortex and the hippocampus following chronic social isolation stress in mice (Ieraci *et al.,* 2016) or chronic restrains stress (Ons *et al.,* 2010). In contrast, increased levels were found in rats following social defeat (Coppens *et al.,* 2011) and in mice subjected to chronic unpredictable mild stress (Boulle *et al.,* 2014).

Pharmacological studies also provide divergent findings. Acute administration of the SSRI drug paroxetine in combination with WAY100,635 caused a region-specific, 5-HT-mediated increase in *Arc* expression in the rat cortical areas (Castro *et al.,* 2003b). Chronic fluoxetine treatment stimulates *Arc* mRNA expression in the cingulate and orbital frontal cortices in rats (De Foubert *et al.,* 2004), though no changes (De Foubert *et al.,* 2004) or increases (Alme *et al.,* 2007; Ferrés-Coy *et al.,* 2016) are found in the hippocampus in rodents. Fast-acting antidepressants (i.e. ketamine and electroconvulsive therapy) acutely increase *Arc* expression (de Bartolomeis *et al.,* 2013; Dyrvig *et al.,* 2012; Larsen *et al.,* 2005). Chronic treatment with agomelatine normalized CUMS-induced increases in the levels of *Arc* mRNA in the hippocampus (Boulle *et al.,* 2014).

mTOR. mTOR is a serine/threonine protein kinase activated by phosphorylation in response to growth factors (for example BDNF). It plays an essential role in the regulation of protein synthesis, energy metabolism, lipid metabolism, cell growth and size, autophagy, lysosome biogenesis, axonal sprouting, axonal regeneration, myelination, dendritic spine growth, etc (reviewed by Réus *et al.,* 2015).

Major depression disorder subjects showed a reduction in mTOR, p70S6K, eIF4B, and p-eIF4B protein expression in the prefrontal cortex (Jernigan *et al.,* 2011). Similarly, blood levels of mTOR mRNA were reduced in bipolar patients during depressive episodes (Machado-Vieira, *et al.,* 2015). Furthermore, ketamine administration was associated with an increase in the plasma levels of mTOR in some depressive patients (Yang *et al.,* 2013).

Rodents exposed to chronic unpredictable mild stress presented a reduction in phosphorylation levels of mTOR, and its phosphor-p70S6K, in the prefrontal cortex, hippocampus, and amygdala (Chandran *et al.,* 2013; Zhong *et al.,* 2014; Zhu *et al.,* 2013). The genetic deletion of mTOR in mice induced a depressive-like behaviour (Zhong *et al.,* 2014). Moreover, mTOR is required for the antidepressant effects of fluoxetine, ketamine and deep brain stimulation in animal models of depression (Holubova *et al.,* 2016; Li *et al.,* 2010; Liu *et al.,* 2015; Jiménez-Sánchez *et al.,* 2016). Therefore, mTOR seems to play a crucial role in the development and the treatment of the depressive disorder.

β-catenin. β-catenin presents two principal functions: a regulatory role in the cytoplasm and nucleus and a structural function at the membrane level, modulates adult neurogenesis and synaptic plasticity (reviewed by Pilar-Cuéllar *et al.,* 2014). Therefore, β-catenin might play an important role in the development of depression and in the mechanism of action of the antidepressant drugs.

Clinical studies report reduced β-catenin levels in human prefrontal cortex samples from depressed subjects (Karege *et al.,* 2012). A decreased levels of β-catenin expression have also been found in animal models of depression in the medial prefrontal cortex and hippocampus (Chen *et al.,* 2012; Liu *et al.,* 2012). Consistently, the β-catenin knocking out in the forebrain showed an increased behavioural despair in the tail suspension test in mice (Gould *et al.,* 2008). Chronic administration of classical antidepressant drugs (Mostany *et al.,* 2008) and other antidepressants strategies (Madsen *et al.,* 2003; Pascual-Brazo *et al.,* 2012; Wexler *et al.,* 2008) increase β-catenin protein and mRNA levels in the subgranular zone of the dentate gyrus. The inhibition of GSK-3β was reported to be necessary for the antidepressant effect of ketamine in the learned helplessness (Beurel *et al.,* 2011).

Neurogenesis. The generation of neurons in the hippocampal dentate gyrus is implicated in both the pathophysiology and the mechanism of action of antidepressant drugs (Petrik *et al.,* 2011). In the 90s it was described that stress, one of the most important risk factor of depression, reduces the generation of hippocampal neurons in rats (Gould *et al.,* 1992). Some years later, it was shown that chronic treatment with antidepressant drugs increased hippocampal proliferation and neurogenesis in

rats and depressed patients (Boldrini *et al.,* 2009; Malberg *et al.,* 2000). An increase of hippocampal proliferation and neurogenesis was also observed after electroconvulsive shock and running therapies in rodents (Madsen *et al.,* 2000; Van Praag *et al.,* 1999). Moreover, the time required for the maturation of these newborn neurons coincides with the timeframe that antidepressant drugs need to induce their therapeutic effects (Petrik *et al*, 2011). However, there are preclinical findings suggesting that neurogenesis is not always required for the antidepressant effects (David *et al.,* 2009; Mendez-David *et al.,* 2014; Santarelli *et al.,* 2003; Surget *et al.,* 2008), suggesting a more complex relationship between this neuroplasticity mechanism and the antidepressant response. Moreover, it is unclear, if a decrease in the neurogenesis could cause a depressive state since in several studies, the ablation of neurogenesis did not modify the levels of anxiety and depression in both *naïve* rodents and animal models of depression (Petrik *et al.,* 2011).

4.2.4. Neuroendocrine hypothesis

Around 50% of depressed patients (80% in severe depressed) present hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis (Holsboer, 2000; Pariante, 2003; Pariante and Miller, 2001), which can be due to a deficit in the negative feedback regulation of the axis (Dam, 1988). Several studies have reported that the normalization of the HPA axis is associated with the clinical response to the antidepressant treatment (see review Binder *et al.,* 2009). The relationship between HPA axis dysregulation and the major depressive disorder is also demonstrated in animal models such as the corticosterone model of depression. The chronic administration of the stress hormone corticosterone in rodents induces a depressive/anxious like state as well as several neurochemical and molecular alterations that resemble those observed in depressive patients (Gourley and Taylor, 2009; Krishnan and Nestler, 2008). Furthermore, due to the relevance of the HPA axis hyperactivity in the major depressive disorder, several molecules that modulates this axis have been developed and tested, but all of them have failed in the clinical trials (Spierling and Zorrilla, 2017).

4.2.5. Other hypothesis: glutamatergic and neuroinflammatory

In the recent years, new hypotheses have been postulated to further understand the pathophysiology of depression. A full description is beyond the scope of this thesis, and we will mention only two of them. First, the glutamatergic hypothesis, which was recently proposed after discovering that NMDA antagonists such as ketamine presented a fast antidepressant action. This hypothesis suggests that the glutamatergic system is a primary mediator of the depressive disorder and a final common pathway for the therapeutic actions of many antidepressant compounds (reviewed by Sanacora *et al.,* 2012). Second, the neuroinflammatory hypothesis since depressed patients show increased levels of circulating cytokines and activation of the brain immune cell microglia (reviewed by Brites and Fernandes, 2015).

4.3. Antidepressant drugs

In the last 50 years, depression treatment has been monopolized by several drugs, which act on the monoamine systems. The serendipitous discovery of the antidepressant properties of iproniazid (a non-selective and irreversible monoamine oxidase inhibitor, MAOI) started a new era in the treatment of depression. Few years later, the antidepressant actions of imipramine (a tricyclic compound developed as a neuroleptic drug) were also discovered. The MAOIs (such as iproniazid and phenelzine) increase monoamine levels by preventing their degradation by monoamine oxidases; however, they present severe side effects such as hypertensive crisis and serotonergic syndrome, and are poor tolerated. Tricyclic antidepressant drugs, such as imipramine or amitriptyline, which block noradrenaline and serotonin reuptake, were the most commonly used antidepressants until the nineties. However, their use has been reduced due to their anticholinergic and cardiovascular adverse effects (Castro *et al.,* 2014a).

In the 1980s, the selective serotonin reuptake inhibitor (SSRIs) appeared, being the most prescribed drugs for the treatment of depression since then. The advantage of SSRIs was their lack of affinity for α-adrenergic, muscarinic, and cholinergic or histaminergic receptors, avoiding the adverse effects typically associated with tricyclic antidepressants. SSRIs are a chemically heterogeneous group that includes

fluoxetine, fluvoxamine, paroxetine, sertraline, citalopram and escitalopram. However, they show two main limitations: the delayed onset of their antidepressant effects and that lack of response of a significant group of patients. The intense research in the field to obtain new antidepressants led to the appearance of selective noradrenaline reuptake inhibitors (i.e. reboxetine), also exhibiting low affinity for different types of receptors and few side effects (Castro *et al.,* 2014a).

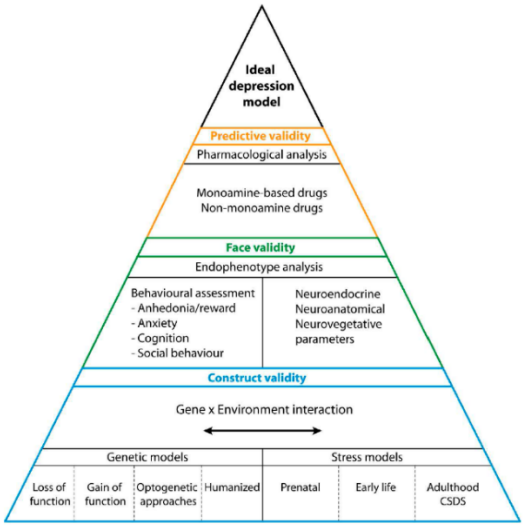
Convincing evidences supported the involvement of both serotonergic noradrenergic system in the etiology of depression, which led to the introduction of a new group of antidepressants known as dual inhibitors of serotonin and noradrenaline reuptake, including venlafaxine and duloxetine. This class of drugs shares with the tricyclic group the property of inhibiting the reuptake of serotonin and noradrenaline but they produce fewer side effects because they have no affinity for muscarinic and adrenergic receptors (Castro *et al.,* 2014a). Approximately 10 years ago, agomelatine appeared. This drug is a melatonine agonist and an antagonist of $5HT_{2C}$ receptors that does not exhibit some of the adverse effects of SSRIs and has a similar efficacy (Castro *et al.,* 2014a). In September of 2013, the FDA approved vortioxetine for the treatment of major depressive disorder. This drug is a 5-HT_{1A} receptor agonist, 5-HT_{1B} receptor partial agonist, 5-HT_{3A} and 5-HT_7 receptor antagonist, and a potent serotonin reuptake inhibitor. Additionally, vortioxetine presented a similar efficacy than classical antidepressant and had lower risk for sexual dysfunction and weight gain than SSRIs (Hillhouse and Porter, 2015). Recently, vilazodone, a compound that is 5-HT reuptake inhibitor and 5-HT_{1A} receptor partial agonist, was also approved by FDA. Some clinical trials suggested that vilazodone could alleviate depressive symptoms after 1 week of treatment, exhibiting a faster onset of action than classical antidepressants (Wang *et al.,* 2016b).

The authentic revolution in the depression treatment appeared with the fast rapid antidepressant effects of ketamine. This NMDA antagonist induces an antidepressant effect in hours and has effect in treatment resistant depression (Zarate *et al.,* 2006; Niciu *et al.,* 2014). Despite the impressive antidepressant properties of this compound, ketamine is not used in the clinical practice due to the safety issues of long-term administration such as the psychotomimetic effect, cognitive impairment, dependence and abuse (Mathew *et al.,* 2012).

5. ANIMAL MODELS OF DEPRESSION

Animal models of depression are valuable tools to understand the pathophysiological mechanisms of major depressive disorder and to evaluate potential antidepressant drugs. These animal models try to resemble the genetic, behavioural, neurochemical and molecular features encountered in depressive patients. Unfortunately, not all the manifestations of depression in humans can be fully recapitulated in a single animal model; indeed, some symptoms of depression cannot be modelled such as guilt, suicidality and sad mood. However, other manifestations such as anhedonia, helplessness, and neurovegetative changes, appetite disturbances and altered sleep patterns are relatively easy to be induced in rodents (Krishnan and Nestler, 2011).

As shown in the pyramid (Dedic et *al.,* 2011), animal models should exhibit three basic features: face validity (phenotype analogous to humans who have the disease), predictive validity (sensitivity to pharmacological interventions that are effective for the disorder in humans) and construct validity (presented similar causatives factors than the human pathology) (Wang *et al.*, 2017). To reach this aim, it will be necessary to combine all recent advances in genetics, pharmacology and electrical stimulation with environmental insults. This would simulate gene-environment-interactions that reflect the pathophysiological mechanisms of major depressive disorder and would help in the research of new antidepressant strategies.



There are several animal models of depression, some induced by acute or chronic stress exposure (chronic unpredictable mild stress, maternal deprivation, learned

helplessness), exogenous administration of glucocorticoids (corticosterone model), genetic manipulations, surgical manipulation (OBX, olfactory bulbectomy), inflammation (LPS, lipopolysaccharide) or social isolation. In the present thesis, we have used the chronic administration of corticosterone and the bilateral olfactory bulbectomy models in mice.

5.1. Corticosterone model

Although the relationship between stress and major depressive disorder is not completely known, it is assumed that stressful life events could induce, or exacerbate depressive episodes (De Kloet, 2004). The corticosterone model of depression consists in the chronic exposition (usually in the drinking water for 4 weeks) to the stress hormone corticosterone. It aims to resemble the neuroendocrine-induced brain and behavioural alterations associated with the chronic stress since the sustained glucocorticoid administration produces similar corticosterone levels than physical and psychological stressors (Sapolsky *et al.,* 1984; Sapolsky *et al.,* 1985; Tornello *et al.,* 1982). It has also the advantage of a lower inter-individual variability than other models based on environmental stress manipulations such as the chronic mild stress (CMS) and learned helplessness, which are more difficult to replicate between laboratories (Matthews *et al.,* 1995; Vollmayr and Henn, 2001; Willner, 2005). For this reason, it is accepted that the corticosterone model endows construct validity, reliable predictive validity and good reproducibility. From a behavioural point of view, this model exhibits a constellation of anxious/depressive-like features. Corticosterone-treated animals show behavioural despair in the forced swimming test, anhedonia-like behaviour in the sucrose test (David *et al.,* 2009; Gourley *et al.,* 2008), decreased grooming (David *et al.,* 2009), suppressed sexual performance (Gorzalka *et al.,* 2001), and decreased food-reinforced responses (Gourley *et al.,* 2008). In addition, chronic corticosterone administration also enhances anxiety responses in several paradigms such as the predator odor (Kalynchuk *et al.,* 2004), light/dark box (Murray *et al.,* 2008), escape behaviour (Stone *et al.,* 1988), open field (David *et al.,* 2009) and novelty suppression feeding (David *et al.,* 2009) tests. Physiological changes resemble those observed in depressed patients such as decreased adrenal weight (Murray *et al.,* 2008), dysregulation of HPA axis function and reduction of weight (Johnson *et al.,* 2006). Locomotor activity during the dark

cycle is disrupted after chronic corticosterone administration, which reflects an impaired circadian rhythm (Gourley and Taylor, 2008). All the above manifestations are similar to those observed in depressed patients, which reinforces the face validity of the model. Moreover, and quite relevant from a pharmacological point of view, the corticosterone-induced behavioural changes can be reversed with chronic antidepressant treatment (David *et al.,* 2009), highlighting the predictive validity of this model.

In addition to the extensive behavioural changes seen in the corticosterone model, there are also neurochemical and molecular alterations. For example, chronic corticosterone administration induces a desensitization of 5-HT_{1A} autorreceptors, though the serotonin levels in the dorsal raphe nucleus are not altered (Hensler *et al.,* 2007; Rainer *et al.,* 2012). There are also several neuroplasticity changes induced by the chronic corticosterone administration: impaired hippocampal neurogenesis (David *et al.,* 2009; Mayer *et al.,* 2006; Murray *et al.,* 2008), dendritic atrophy in the hippocampus (Magarinos *et al.,* 1999; Sousa *et al.,* 2000) and prefrontal cortex (Cerqueira *et al.,* 2005; Wellman, 2001), increases in dendritic morphology in the amygdala (Mitra and Sapolsky, 2008), and decreases of brain derived neurotropic factor (BDNF) in the hippocampus and prefrontal cortex (Dwivedi *et al.,* 2006; Jacobsen and Mork, 2006).

5.2. Olfactory bulbectomy

Surgical removal of the olfactory bulbs in rodents leads to behavioural, neurotransmitter, neuroendocrine and immune changes resembling those found in depressed patients (Song and Leonard, 2005). Besides face validity, the olfactory bulbectomy model (OBX) presents a high predictive validity of antidepressant activity following chronic administration.

The characteristic behavioural phenotype of the olfactory bulbectomy model is the locomotor hyperactivity (Song and Leonard, 2005), proposed to mimic psychomotor agitated depression (Lumia *et al.,* 1992). This hyperactivity in the open field is one of its main manifestations that is reversed by chronic, but not acute, treatment with antidepressants, which models the time needed to observe the beneficial effects of clinically used antidepressants. In fact, the attenuation of the hyperactivity in OBX

rodents is used as a predictive marker when testing putative antidepressant compounds (Hendriksen *et al.,* 2015). Olfactory bulbectomized animals also exhibit anhedonia, altered social behaviour, impaired learning and memory capacities, decreased sexual behaviour and anxiety (See review by Roche, 2009).

Not all these behavioural alterations can be explained by the anosmia since they are the result of the neuronal reorganization in cortical-hippocampal-amygdala circuits following the surgery (Song and Leonard, 2005). Olfactory bulbectomy induces neuronal atrophy and remodelling in the cortex, amygdala, hippocampus, raphe nuclei and *locus coeruleus* (Carlsen *et al.,* 1982; Nesterova *et al.,* 1997; Norrholm and Ouimet, 2001). There are several adaptive changes in the serotonergic system associated with the behavioural manifestations. 5-HT and 5-HIAA levels are reduced after the surgery in the frontal cortex (Redmond *et al.,* 1997), amygdala and hippocampus (van der Stelt *et al.,* 2005). A reduction in the levels of tryptophan hydroxylase and consequently a decreased rate of 5-HT synthesis is detected in OBX mice (Hellweg *et al.,* 2007). Studies on the regulation of the functionality and density of 5-HT_{1A} receptors after the olfactory bulbectomy have yielded different results. Our group has described a reduction in the 5-HT_{1A} functionality, measuring the stimulation of G-proteins after 5-HT_{1A} receptor activation by autoradiographic means, in several brain areas: dorsal raphe nucleus, hippocampus and amygdala of OBX (Linge *et al.,* 2016). Previous studies described no changes in the density (Gurevich *et al.,* 1993) and functionality (McGrath and Norman, 1998) of 5-HT_{1A} receptors after OBX surgery. A recent study showed an increased cell surface expression of 5-HT_{1A} autoreceptors by olfactory bulbectomy and its reversion after chronic fluoxetine treatment (Riad *et al.,* 2017). Moreover, olfactory bulbectomy also induces alterations in some brain neuroplasticity markers, and especially in the hippocampus, that, in some cases are specie-dependent (Hendriksen *et al.,* 2015). In mice, BDNF levels in the hippocampus are increased in OBX mice (Freitas *et al.,* 2013; Hellweg *et al.,* 2007), but decreased in OBX rats (Hendriksen *et al.,* 2012). Hence, the resultant neurochemical and molecular alterations appear to alter the functioning of some brain areas implicated in many of the behavioural responses of OBX animals.

"*El científico no es aquella persona que da las respuestas correctas, sino aquél quien hace las preguntas correctas.*"

Claude Lévi-Strauss

II. HYPOTHESIS AND OBJECTIVES

Based on the previous clinical and animal studies, we hypothesised that the deletion of brain 5-HT_4 receptors could be associated with a depressive- and anxiety-phenotype. This genetic trait would increase the vulnerability in the adulthood to show those "pathological" manifestations mimicking human depression/anxiety disorders. In addition, we also hypothesized that the deletion of 5-HT_4 receptors would influence the pharmacological actions of RS67,333, a partial 5-HT_4 agonist, and a classical SSRI antidepressant.

The main *objective* was to provide further knowledge regarding the role of 5-HT_4 receptors in anxiety/depression disorders and in the mechanism of action of the antidepressants drugs, with the aid of a constitutive 5-HT_4 receptor *knockout* mice.

To answer the above hypotheses, the following objectives were proposed:

1. To characterize the behavioural (paradigms of anxiety and depression), neurochemical (functionality of 5-HT_{1A} receptors) and molecular (BDNF, trkB and *Arc* mRNA expression as well as the levels of mTOR and β-catenin and their phosphorylated forms) phenotypes of 5-HT_4 receptor *knockout* mice in comparison with *wild type* mice.

2. To compare the behavioural, neurochemical and molecular phenotypes of 5-HT_4 receptor *knockout* and *wild type* mice when they subjected to two "pathological" animal models of depression: the chronic administration of corticosterone model and the bilateral olfactory bulbectomy.

3. To evaluate the behavioural, neurochemical and molecular effects induced by the chronic treatment with the 5-HT_4 partial agonist RS67,333 and the SSRI fluoxetine in 5-HT_4 receptor *knockout* mice and the respective *wild type* counterparts, and in both animal models of depression.

"*Si buscas resultados distintos,*
no hagas siempre lo mismo."

Albert Einstein

III. Material and methods

1. ANIMALS

Wild-type (WT) and 5-HT$_4$ KO mice were kindly given by Valerie Compan (Compan *et al*., 2004). 5-HT$_4$ KO mice were generated inserting the gen enconding for the neomycin phosphotransferase (Neo), under the control of the phosphoglycerate kinase I promoter in the exon III of the 5-HT$_4$ gene that codifies the third transmembrane domain (Fig. 6) (detailed protocol of the generation of the 5-HT$_4$ KO mice in Compan *et al*., 2004). This genetic insertion created a disruption of the open reading frame and blocked the expression of the gene (Hall *et al*., 2009). The 5-HT$_4$ KO and wild-type mice (3 months old, 25 ± 1 g) were obtained from the breeding of 5-HT$_4$ heterozygote 129SvTer mice (Compan *et al*., 2004) or 5-HT$_4$ KO mice and were housed (n = 4-5 per cage) in the animal house of the University of Cantabria. They were kept in a temperature (21 ± 0.1°C), controlled environment with 12 h light/dark cycle, and with food and water available *ad libitum* (unless otherwise mentioned for some behavioural tests). All WT and 5-HT$_4$ KO mice used in this thesis were genotyped (see detailed protocol below) before starting the experimental procedures.

All the experiments were carried out with the approval of the Animal Care Committee of the Universidad de Cantabria, and according to the Spanish legislation (Real Decreto 53/2013) and the European Communities Council Directive 2010/63/UE on "Protection of Animals Used in Experimental and Other Scientific Purposes".

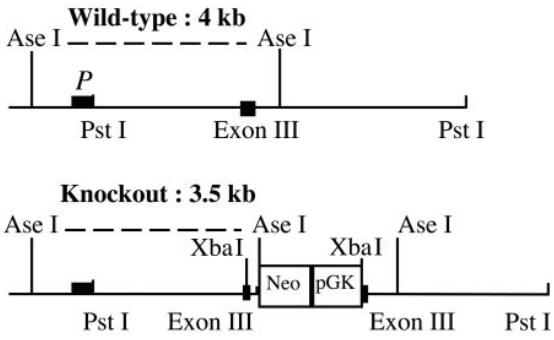


Figure 6. Schematic representation of the 5-HT$_4$ KO mice construct. As shown in the picture, the neomycin cassette was inserted in the exon III and disrupted the exon (Compan *et al*., 2004).

2. EXPERIMENTAL DESIGN AND PHARMACOLOGICAL TREATMENTS

2.1. Experimental design

Different studies were designed depending on each specific aim of the thesis. In each one, the experimental groups, and the number of animals *per* group, were determined according to the type of study and the requirements of each technique. The number of subjects *per* group (n) is indicated in the figure captions.

In the first study, *naïve* WT and 5-HT$_4$ KO animals were used to carry out the adenylate cyclase assays. The functionality of the 5-HT$_4$ receptor (see detailed protocol below) was evaluated to confirm the absence of this receptor in KO mice.

The second study was aimed to compare the phenotype of *naïve* WT- and KO-mice using behavioural, neurochemical and molecular approaches:

1. A first set of mice were subsequently subjected to several tests assessing anxiety- and depression-related behaviours (Fig. 7).

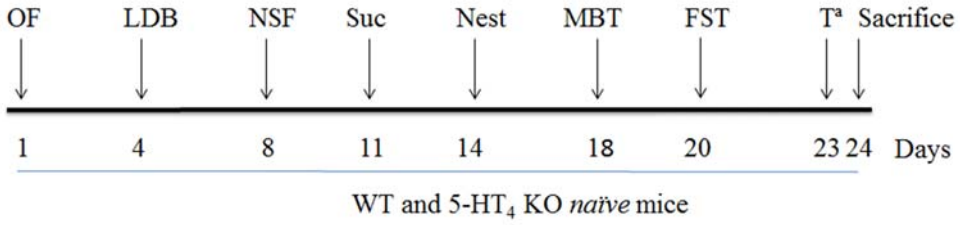


Figure 7. Chronogram of the experiments carried out in WT and 5-HT$_4$ KO *naïve* mice. OF: open field test, LDB: light/dark box test, NSF: novelty suppressed feeding, Suc: sucrose intake test, Nest: nesting test, MBT: marble burying test, FST: forced swimming test, Tª: (+)-8-OH-DPAT induced hypothermia.

2. A separate set of *naïve* WT- and KO-mice were sacrificed to carry out the [^{35}S]GTPγS autoradiography of 5-HT$_{1A}$ receptors, *in situ* hybridization studies of BDNF, trkB and *Arc* mRNA expression, and western blot assays of pmTOR/mTOR and p-β-catenin/β-catenin protein expression in the hippocampus.
3. A third set of *naïve* WT- and KO-mice was used for the assays of hippocampal proliferation (immunohistochemistry of BrdU).

Finally, in the third study, WT and 5-HT$_4$ KO mice were subjected to two different animal models of depression/anxiety: the chronic administration of corticosterone (CORT) and the bilateral olfactory bulbectomy (OBX) (section 6). Then, we compared the effects of the treatment with RS67,333 and fluoxetine in each model (CORT, see Figs. 8 and 9; OBX, see Figs. 10 and 11). For this purpose, the behavioural testing was done at different time-points along the treatment, and always 24 hours after the last dose and before administering the next one. After completion of the behavioural phenotyping, mice were sacrificed to carry out the neurochemical and molecular studies mentioned above.

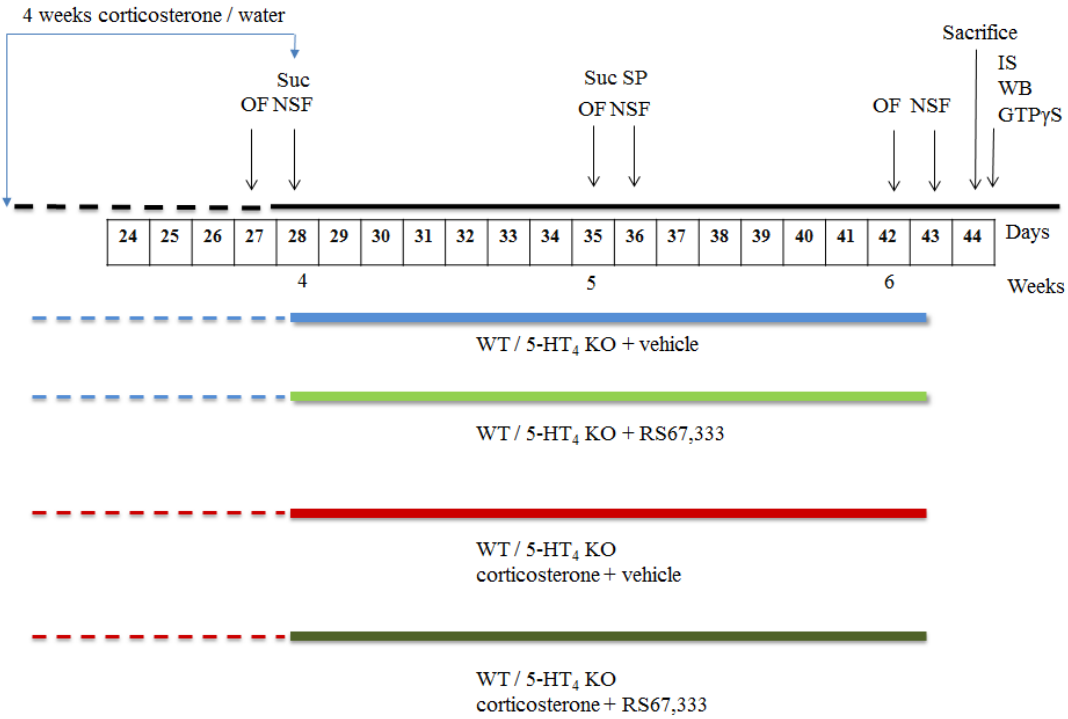


Figure 8. Chronogram of the chronic treatment with RS67,333 (1.5 mg/kg/day, i.p.) or vehicle (saline, i.p.) in the CORT model. OF: open field, NSF: novelty suppressed feeding, Suc: sucrose intake, SP: splash test, IS: *in situ* hybridization, WB: western blot, GTPγS: [^{35}S]GTPγS autoradiography of 5-HT$_{1A}$ receptor.

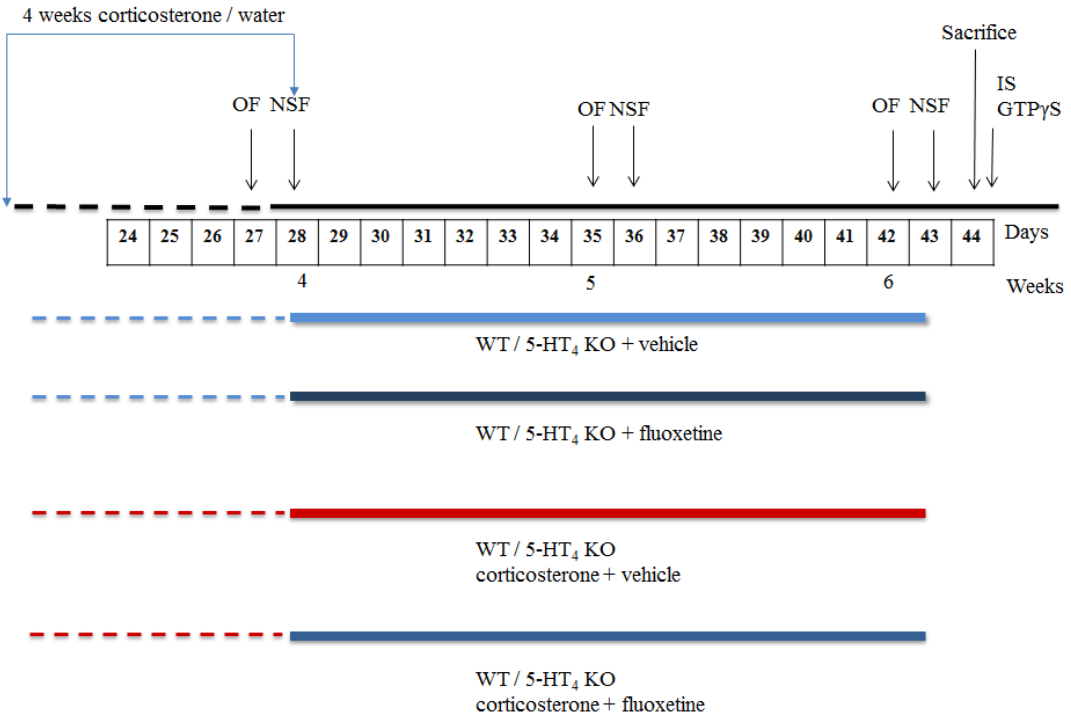


Figure 9. Chronogram of the chronic treatment with fluoxetine (25 mg/kg/day, orally) or vehicle (drinking water) in the CORT model. OF: open field, NSF: novelty suppressed feeding, IS: *in situ* hybridization, WB: western blot, GTPγS: [^{35}S]GTPγS autoradiography of 5-HT$_{1A}$ receptor.

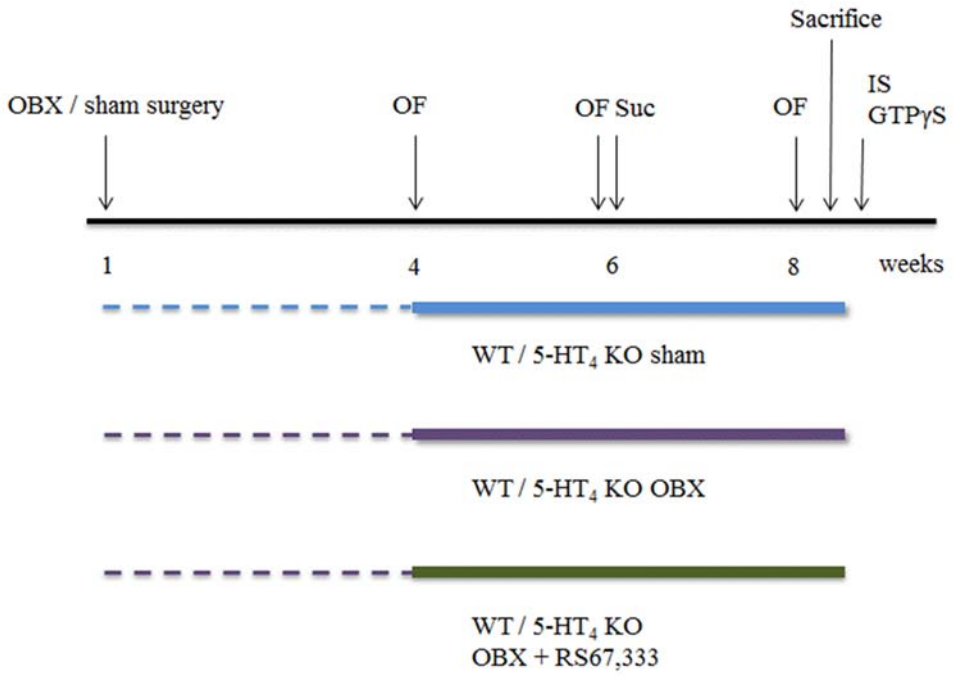


Figure 10. Chronogram of the chronic treatment with RS67,333 (1.5 mg/kg/day, i.p.) or vehicle (saline i.p.) in the OBX model. OF: open field, Suc: sucrose intake, IS: *in situ* hybridization, WB: western blot, GTPγS: [^{35}S]GTPγS autoradiography of 5-HT$_{1A}$ receptor.

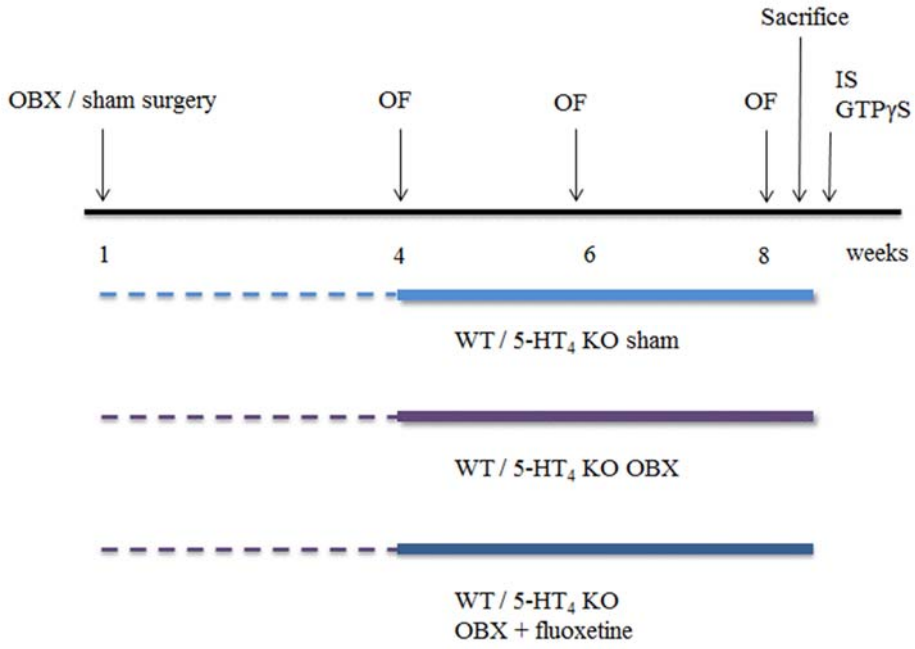


Figure 11. Chronogram of the chronic treatment with fluoxetine (25 mg/kg/day, orally) or vehicle (drinking water) in the OBX model. OF: open field, IS: *in situ* hybridization, WB: western blot, GTPγS: [^{35}S]GTPγS autoradiography of 5-HT$_{1A}$ receptor.

2.2. Pharmacological treatments

2.2.1. Chronic treatment with RS67,333 or fluoxetine

The partial 5-HT$_4$ agonist RS67,333 (1.5 mg/kg/day, once a day) or its vehicle (saline) were administered (i.p.; 0.1 ml/10 g of body weight) for the time indicated in the respective chronograms of corticosterone and OBX models. The dose of RS67,333 was selected according to previous studies in the literature (Mendez-David *et al*., 2014; Vidal *et al*., 2013). A stock solution was prepared every 1 week and kept at -20ºC until use. Each day it was thawed at room temperature and diluted with saline to be administered between 10.00 and 14.00 a.m.

The selective serotonin reuptake inhibitor (SSRI) fluoxetine was administered in the drinking water (160 mg/l, equivalent to a dose of 25 mg/kg/day). Fluoxetine solutions were given in the standard animal house bottles, and were replaced every 1 week. Consumption was monitored every other day and, if necessary, the concentration was corrected to administer the right dose.

2.2.2. Chronic administration of corticosterone

The corticosterone hemisuccinate was dissolved in commercial still mineral water with low mineral content (Corconte®) and solved at pH = 12 (around the pK_b of the molecule). The basic solution was stirred for 6 hours at 4ºC in a dark room and then was neutralized (pH = 7.4) with HCl 10N. A low dose of corticosterone was administered in the drinking water (45 mg/l, equivalent to a dose of 7-10 mg/kg/day) in opaque bottles. Corticosterone solutions were changed every 7 days to avoid degradation. As indicated for fluoxetine solutions, consumption of corticosterone was monitored and, if necessary, the concentration was corrected to administer the right dose. In the groups of animals receiving fluoxetine, the antidepressant was added to the corticosterone solution at the appropriate concentration.

3. DRUGS AND REAGENTS

The 5-HT_4 agonist zacopride (10 µM in ultrapure Elix® water) was freshly prepared for the adenylate cyclase assays. The 5-HT_{1A} agonist (±)-8-OHDPAT was freshly prepared for the [^{35}S]GTPγS autoradiography of 5-HT_{1A} receptors (1 mM in ultrapure Elix® water). The (+)-8-OH-DPAT (1 mg/kg; i.p.; in saline) was used in the *in vivo* assay of 5-HT_{1A} receptor-mediated hypothermia. Isofluorane (induction: 4% and maintenance: 2%) was used as inhaled anaesthetic (in 100% O_2 at a 1.5-2 litres/min flow) for the olfactory bulbectomy surgery.

For the hippocampal proliferation studies, BrdU (5-Bromo-2´-Deoxyuridine) was administered i.p. (75 mg/kg; four injections every two hours) a day before sacrifice. Pentobarbital sodium (50 mg/kg, i.p.) was used as a systemic anaesthetic to carry out the transcardial perfusion of animals with saline and paraformaldehyde.

All other chemical and reagents were of analytical grade. The chemicals, drugs, reagents and other items, and the commercial suppliers, are listed in table 1.

Table 1. Drugs/reagents and suppliers

Name	**Suppliers**
(+)-8-OHDPAT ((2R)-(+)-8-Hydroxy-2-(di-n-propylamino)tetralin hydrobromide	Tocris Bioscience, Bristol, United Kingdom
(±)-8-OHDPAT ((±)-8-Hydroxy-2-(di-n-propylamino)tetralin hydrobromide	Sigma-Aldrich Química SL, Madrid, Spain
[^{35}S]-dATP (2' Deoxyadenosine 5'-(α-thio) Triphosphate)	Perkin Elmer, Massachusetts, USA
[^{35}S]-GTPγS ([^{35}S] Guanosine 5'-(γ-thio) Triphosphate)	Perkin Elmer, Massachusetts, USA
3-Isobutylmethylxanthine	Sigma-Aldrich Química SL, Madrid, Spain
Acetic acid	Sigma-Aldrich Química SL, Madrid, Spain
Acetic anhydride	Sigma-Aldrich Química SL, Madrid, Spain
Acrylamide/Bis Solution	Bio-Rad Laboratories SA, Alcobendas, Spain
Adenosine deaminase	Sigma-Aldrich Química SL, Madrid, Spain
Agarose	Sigma-Aldrich Química SL, Madrid, Spain
Ammonium persulfate (APS)	Sigma-Aldrich Química SL, Madrid, Spain
Bone Wax	B. Braun, Barcelona, Spain
BrdU (5-Bromo-2´-Deoxyuridine)	Fischer Scientific SL, Madrid, Spain
BSA (bovine serum albumine)	Sigma-Aldrich Química SL, Madrid, Spain
Chloroform	Sigma-Aldrich Química SL, Madrid, Spain
Corticosterone hemisuccinate (4-Pregnen-11β, 21-diol-3, 20-dione 21-hemisuccunate)	Steraloids Inc., Rhode Island, USA
Creatine phosphokinase	Sigma-Aldrich Química SL, Madrid, Spain
DEPC (Diethyl pyrocarbonate)	Sigma-Aldrich Química SL, Madrid, Spain
Dextran sulphate	Sigma-Aldrich Química SL, Madrid, Spain
Dream TAQ Green PCR Master Mix	Fischer Scientific SL, Madrid, Spain

Name	**Suppliers**
DTT (1,4-Dithiothreitol)	Sigma-Aldrich Química SL, Madrid, Spain
EDTA (Ethylendiaminetetraacetic acid)	Sigma-Aldrich Química SL, Madrid, Spain
EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid)	Sigma-Aldrich Química SL, Madrid, Spain
Elix® ultrapure water	Millipore SAS, Molsheim, French
Ethanol	Scharlab SL, Barcelona, Spain
Ethylene glycol	Sigma-Aldrich Química SL, Madrid, Spain
Fluoxetine hydrochloride	Tocris Bioscience, Bristol, United Kingdom
Formamide deionized	Sigma-Aldrich Química SL, Madrid, Spain
GDP (Guanosine 5′-diphosphate sodium salt)	Sigma-Aldrich Química SL, Madrid, Spain
Glycerol	Sigma-Aldrich Química SL, Madrid, Spain
Glycine	Sigma-Aldrich Química SL, Madrid, Spain
Goat serum	Sigma-Aldrich Química SL, Madrid, Spain
GTPγS (Guanosine 5'-(γ-thio) Triphosphate tetralithium salt)	Sigma-Aldrich Química SL, Madrid, Spain
Guanidine hydrochloride	Sigma-Aldrich Química SL, Madrid, Spain
HCl	Sigma-Aldrich Química SL, Madrid, Spain
Heparin	Sigma-Aldrich Química SL, Madrid, Spain
Isoflurane	Schering-Plough SA, Alcobendas, Spain
Isopropanol	Sigma-Aldrich Química SL, Madrid, Spain
Leupeptin	GE Healthcare Europe GmbH, Barcelona, Spain
Lidocaine	B. Braun, Madrid, Spain
Methanol	Scharlab SL, Barcelona, Spain
Mg-ATP (Adenosine 5′-triphosphate magnesium salt)	Sigma-Aldrich Química SL, Madrid, Spain
$MgCl_2.6H_2O$	Sigma- Aldrich Química SL, Madrid, Spain

Name	**Suppliers**
Milli-Q® ultrapure water	Millipore SAS, Molsheim, France
Myokinase	Sigma-Aldrich Química SL, Madrid, Spain
Na_2HPO_4	Sigma-Aldrich Química SL, Madrid, Spain
NaCl	Sigma-Aldrich Química SL, Madrid, Spain
NaH_2PO_4	Sigma-Aldrich Química SL, Madrid, Spain
NaOH	Sigma-Aldrich Química SL, Madrid, Spain
Paraformaldehyde	Sigma-Aldrich Química SL, Madrid, Spain
PBS	Sigma-Aldrich Química SL, Madrid, Spain
Pentobarbital sodium	Vetoquinol E.V.S.A., Madrid, Spain
Phenol red	Sigma-Aldrich Química SL, Madrid, Spain
Phosphocreatine	Sigma-Aldrich Química SL, Madrid, Spain
Polivinilpirrolidone	Sigma-Aldrich Química SL, Madrid, Spain
Polyadenylic Acid (Poly A)	Sigma-Aldrich Química SL, Madrid, Spain
Proteinase K	Sigma-Aldrich Química SL, Madrid, Spain
RS67,333 hydrocloride	Tocris Bioscience, Bristol, United Kingdom
Salmon sperm DNA	Sigma-Aldrich Química SL, Madrid, Spain
SDS (Sodium dodecyl sulfate)	Fischer Scientific SL, Madrid, Spain
Sodium citrate	Sigma-Aldrich Química SL, Madrid, Spain
Sodium phosphate	Sigma-Aldrich Química SL, Madrid, Spain
Sodium pyrophosphate	Sigma-Aldrich Química SL, Madrid, Spain
Sodium vanadate	Sigma-Aldrich Química SL, Madrid, Spain
Sucrose	Scharlab SL, Barcelona, Spain
SYBR® safe DNA gel stain	Fischer Scientific SL, Madrid, Spain
TEA (Triethanolamine)	Sigma-Aldrich Química SL, Madrid, Spain

Name	**Suppliers**
TEMED (Tetramethylethylenediamine)	Sigma-Aldrich Química SL, Madrid, Spain
Terminal deoxynucleotide transferase (TdT)	Fischer Scientific SL, Madrid, Spain
Tris-HCl	Sigma-Aldrich Química SL, Madrid, Spain
Triton X	Sigma-Aldrich Química SL, Madrid, Spain
TRIzol	Fischer Scientific SL, Madrid, Spain
Tween 20	Sigma-Aldrich Química SL, Madrid, Spain
Urea	Sigma-Aldrich Química SL, Madrid, Spain
Zacopride hydrochloride ((±)-4-Amino-N-1-azabicyclo[2.2.2]oct-3-yl-5-chloro-2-methoxybenzamide hydrochloride)	Tocris Bioscience, Bristol, United Kingdom

4. GENOTYPING

4.1. DNA extraction

Firstly, 1 cm of tail of mouse pups (1 month old) was cut and mixed with 400 µl of tail buffer (10 mM of Tris pH= 8, 50 mM EDTA, 100 mM of NaCl, 0.5% SDS) and 15 µl of proteinase K. This mix was incubated overnight at a temperature of 55ºC. The next day, 330 µl of tail buffer were added and the solution was manually mixed for 2 minutes. Then, 250 µl of NaCl 6M were added and the samples were manually mixed. Samples were centrifuged for 10 minutes at room temperature at 13000 r.p.m. Then, 750 µl of supernatant were mixed with 500 µl of isopropanol and vortexed for 2 minutes. Samples were centrifuged for 10 minutes at 4ºC at 13000 r.p.m., the supernatant was discarded and 1 mL of ethanol (70%) was added. The mix was centrifuged for 5 minutes at 4ºC at 13000 r.p.m. Then, the ethanol was completely removed and the samples were dried at room temperature for 1 hour. Finally, 500 µl of TE buffer (10 mM Tris and 1 mM EDTA) were added and the mix was incubated for 20 minutes at 55ºC. The DNA extracted was stored at -20ºC.

4.2. PCR amplification and detection of the fragments

The genotype of the mice was detected using polymerase chain reaction (PCR). The pair of forward and reverse primers were designed to amplify wild-type allele (5'TGGCCTATACCTTTTCCCTCAC3' and 5'TTGCTCTGCTCTTACCTGTCC A3') and the KO allele (5'TGGCCTATACCTTTTCCCTCAC3' and 5'TCTCTTGATTCCCACTTTGT GGTT3'). The amplification of WT or 5-HT_4 KO allele were done in separate PCR amplifications. Primers annealing with the WT allele (250 bp) were located inside the sequence. In contrast, in the 5-HT_4 KO allele (350 bp) the reverse primer annealed to the neo-cassette. Two PCR amplifications were done for each mouse genotyped. Each PCR mixture contained: 0.6 µl of each of the corresponding primer (primer concentration was 10 µM), 0.6 µl of $MgCl_2$ (50 mM), 7.5 µl DreamTaq Green PCR Master Mix (contains TAQ polymerase, 0.4 mM of each dNTPS, 4 mM $MgCl_2$, a density reagent and two dyes to follow electrophoresis progress), 1 µl of tail DNA extract and 4.7 µl of ultrapure Milli-Q® water. Reactions were carried out in 2720 thermal cycler (Applied Biosystems, Foster city, USA) using cycling conditions described in table 2. After PCR amplification, 15 µl of the PCR product were electrophoresed in 2% agarose 1× TAE (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) gels. For visualization of PCR products, gels were stained with 1×SYBR® Safe DNA Gel Stain and digital images were captured in Image quant 350 (General Electric, Boston, USA).

Table 2. Thermocycling conditions for detection WT and KO allele.

T ºC	Duration (s)	Number cycle
95	60	1
95	30	2
64	40	
72	60	
95	30	2
61	40	
72	60	
95	30	2
58	40	
72	60	
95	30	2
55	40	
72	60	
95	30	2
52	40	
72	60	
95	30	19
50	40	
72	60	
72	600	1

5. ADENYLATE CYCLASE ASSAY

The adenylate cyclase assays were performed to confirm the lack of functional 5-HT_4 receptors in the KO mice. 5-HT_4 receptor stimulated adenylate cyclase assays were carried out as previously described with slight modifications (Vidal *et al*., 2009).

5.1. Membrane preparation

Striatal tissue samples were homogenised (1:120 w/v) in 20 mM Tris-HCl, 2 mM EGTA, 5 mM EDTA, 320 mM sucrose, 1 mM dithiothreitol (DTT), 25 µg/mL leupeptin, pH = 7.4, with a mechanical stirrer at 800 r.p.m. (RZR 2020, Heidolph Schwabach, Germany). The homogenised preparation was centrifuged at 500 x g for 5 min at 4°C. The supernatants were centrifuged again at 13000 x g for 15 min at 4°C (Eppendorf centrifuge 5417R, Eppendorf, Hamburg, Germany) and the pellets were resuspended in 20 mM Tris-HCl, 1.2 mM EGTA, 0.25 M sucrose, 6 mM $MgCl_2$, 3 mM DTT and 25 µg/mL leupeptin. The membranes were used immediately after the preparation to avoid degradation of adenylate cyclase.

5.2. 5-HT_4 receptor stimulated adenylate cyclase assay

Membrane (25-50 µg of protein) were pre-incubated for 5 min at 37ºC in reaction buffer (75 mM Tris-HCl pH = 7.4, 5 mM $MgCl_2$, 0.3 mM EGTA, 60 mM sucrose, 1 mM DTT, 0.5 mM 3-isobutylmethylxanthine as a phosphodiesterase inhibitor, 5 mM phosphocreatine, 50 U/mL creatine phosphokinase and 5 U/mL myokinase to regulate the cyclic adenosine nucleotides homeostasis) and 25 µl of either water (basal activity) or the 5-HT_4 agonist zacopride (10 µM). The reaction was started by the addition of 0.2 mM Mg-ATP and incubated at 37°C for 10 min. The reaction was stopped by boiling the samples for 4 min and then were centrifuged at 13000 x g for 5 min at 4°C. cAMP accumulation was quantified using a Cyclic AMP Competitive ELISA Kit (Thermo Fisher Scientific, Waltham, USA). Membrane protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Munich, Germany) and BSA as standard.

5.3. Determination of protein concentration

An aliquot of each membrane preparation was used to determine the protein concentration using a Bio RAD DC kit. First, 25 µl of solution A containing copper tartrate in alkaline medium and 200 µl of solution B containing the Folin reagent were added to every protein aliquot. The mixture was incubated in darkness for 5 minutes, during this time copper reacted with some amino acids of the protein and these amino acids with copper reduced Folin producing a product of an intense blue color that can be measured. The absorbance at 620 nm was measured in the Mithras (LB 940, Berthold technologies, Bad Wildbad, Germany). To determine protein concentration, BSA standard curves were used.

5.3. Data and statistical analysis¡Error! Marcador no definido.

The cAMP values were extrapolated from the calibration curve and corrected for the amount of protein of the sample. The data were expressed as % of basal value (considering basal value as 100%). Data were represented as mean ± S.E.M. A two-way ANOVA analyses was performed with genotype and treatment (basal and zacopride) as variables, followed by Newman-Keuls *post-hoc* test. Graphs editing and statistical analyses were performed using the GraphPad Prism 6 version 6.01 for Windows (GraphPad Software, Inc., La Jolla, USA).

6. ANIMAL MODELS OF DEPRESSION

6.1. Corticosterone model

Corticosterone model of depression was adapted from the method previously described (David *et al*., 2009). A low dose of corticosterone (45 mg/l, equivalent to a dose of 7-10 mg/kg/day) was administered in the drinking water for 4 weeks in opaque bottles and were changed every 7 days to avoid degradation. After 4 weeks of treatment, a battery of anxiety and depression-related tests was performed to confirm the development of the depression/anxiety-induced behaviours before the initiation of drug treatments.

6.2. Bilateral olfactory bulbectomy (OBX)

The OBX procedure was performed as previously described (Linge *et al*., 2013). Mice were anesthetized with isoflurane/O_2 mixture (induction: 4% and maintenance: 2%; O_2 at 1.5/2 litres/min) during all the surgical procedure. Additionally, local anaesthesia was provided with an intradermal injection of lidocaine under the scalp. At the beginning of the procedure, the head was shaven and a midline sagittal incision was made in the skin overlying the skull. A burr hole (2 mm of diameter in the midline 1 mm rostral to the sagittal suture) was drilled (with a surgical drill), through which both olfactory bulbs were bilaterally aspired by a suction pump using cannula with a blunted tip. Then, the burr hole was filled with bone wax in order to avoid further bleeding. Finally, after disinfection (with iodide solution), the scalp was closed using surgical suture 3.0 (Suturas Aragó, Barcelona, Spain). Sham operations were done in the same way, although the bulbs were left intact. After the bulbectomy/sham surgery, mice were placed for 24 hours in a recovery room with a controlled temperature of 27ºC. Then, a four-week period was awaited before the initiation of the behavioural experiments and pharmacological treatments in order that mice recovered and the OBX syndrome was developed appropriately. At the end of the study, animals were sacrificed and the lesions were verified to discard frontal pole lesions and/or incomplete removal of olfactory bulbs.

7. BEHAVIOURAL STUDIES

Behavioural tests were performed during the light phase and ordered from the least to the most stressful one. Mice were placed in the experimental room one hour before the start of each experiment to acclimatize, with the exception of the sucrose intake and nesting tests that were carried out in their own home-cages in the housing rooms.

7.1. Open field test

The open field (OF) test was performed to evaluate the locomotor activity and anxiety response to novelty (Amigó *et al*., 2016). The apparatus consisted in a

wooden box (50 cm x 50 cm x 30 cm) with the centre of the arena highly illuminated (400 lux). A central zone (30 x 30 cm) presented higher illumination than the rest of the OF and absence of the protective effects of the walls. For this reason, central activity parameters (time, distance and number of entries in the central zone) are measurements of anxiety-responses in rodents. Mice were placed in a corner of the open field and allowed to freely explore it for 5 min. At the end of each test, the open field was cleaned with ethanol (70%) to remove olfactory clues. Mice behaviour was automatically video-tracked and analysed using the ANY-maze™ tracking software-version 4.99 (Stoelting Co., Wood Dale, USA). The total distance travelled, peripheral distance and central parameters (time spent and number of entries) were measured.

7.2. Light/dark box

The light/dark box test (LDB) was performed as previously described (Clément *et al*., 2009). This test is broadly used to test the anxiogenic or anxiolytic effects of new compounds and/or following genetic modifications. The apparatus consisted of a plexiglas shuttle box purchased to ANY-maze™ (Stoelting Co., Wood Dale, USA), where the chambers (40 cm x 20 cm x 35 cm) were separated by a small door. One chamber was illuminated with a high intensity light (400 lux) whereas the other was dark. The time and number of entries to the bright zone is indicative of the anxiety levels of mice. At the beginning of the test, mice were individually placed on the dark side and allowed to freely explore it for 5 minutes. At the end of every test, the light/dark box apparatus was cleaned with ethanol (70%) to remove olfactory cues. The time and number of entries into each zone were recorded (ANY-maze™ tracking software 4.99, Stoelting Co., Wood Dale, USA).

7.3. Novelty suppressed feeding

The novelty suppressed feeding (NSF) test was performed as previously described (Linge *et al*., 2013). NSF is a conflict-based anxiety test; in fact, mice deprived of food for one day must choose between approaching to the centre of the arena and eating a pellet of food or staying at the periphery to avoid the anxiogenic central zone.

The NSF was performed after a 24-hour food-deprivation period. The next day, each mouse was placed in a corner of the open field (50 cm x 50 cm x 30 cm; luminance 40-50 lux) containing a wood chip bedding (a height of 2 cm) with a food pellet (2 g) placed in the centre, which was replaced after each test to avoid olfactory cues. The latency (in seconds) to eat the pellet was recorded (maximum 10 minutes) with the aid of ANY-maze™ tracking software 4.99 (Stoelting Co., Wood Dale, USA). Immediately after the eating event, the mouse was single placed into the home cage and allowed to feed freely for 5 minutes, and the amount of food consumption was measured (food consumption post-NSF test).

7.4. Sucrose intake test

The sucrose intake test is used to assess the anhedonia-like behaviour in mice. It evaluates the consumption of sugar solutions, which is a pleasant stimulus in mice, as a measure of the brain reward system, and it is widely used to assess the effects of drugs and genetic modifications on the hedonic state. It was performed as previously described (Linge *et al*., 2013). Mice were deprived of any drink solution for 24 hours and subsequently each animal was given free access to a sucrose solution (1%) for 1 hour, and the volume (ml) consumed by each animal were measured. To reduce the amount of liquid wasted, sucrose bottles were placed upside down twelve hours before the test.

7.5. Forced swimming test

The forced swimming test consists in the measurement of distinct behaviours of mice when placed in a water tank from which they are unable to escape. This increases their stress level and leads to a situation of helplessness or behavioural despair that is reflected as immobility. It is the most widely used behavioural assay for the screening of potential antidepressant compounds (Porsolt *et al*., 1977).

The mice were individually placed in a glass cylinder (height 24 cm, internal diameter 12 cm) filled with water at 25°C. The mice were left in the cylinder during a 6 min session. In the last four minutes of the test, total immobility, swimming and climbing time were manually scored by a trained observed in blind conditions using the videotaped FST sessions. The three parameters were defined as previously

reported (Detke *et al*., 1995a). Immobility was considered when mice were floating with minimal movements to keep the head outside the water. Swimming was considered when mice produced movements usually horizontal throughout the glass cylinder and climbing was considered when mice produced active vigorous movements with the forepaws in and out of the water.

7.6. Marble burying test

Marble burying test (MBT) was adapted from Thomas *et al*. (2009). This test evaluates the number of marbles buried by mice. Mice were placed in a cage (40 cm x 20 cm x 20 cm) with the floor filled with abundant bed (a height of 5 cm) and 15 marbles geometrically distributed on it. During the test, the mouse was placed in the cage and allowed to explore it for 30 minutes. At the end of the test, mice were removed from the cage and the number of marbles buried (more than 50% of the marble covered by the cage bed) was counted.

7.7. Nesting test

Nesting test evaluates the construction of nest in mice. The nesting test was adapted from Deacon (2006). In the test day, mice were individually housed and a 5 cm square of cotton were placed in every cage at the beginning of dark phase. After 12 hours, the nest score was evaluated by using the following scale, where 1 represents and intact cotton or no nest built, and 5 score is a nest perfectly built (Deacon, 2006).

7.8. Splash test

The splash test was adapted from the method previously described (David *et al*., 2009). The test consisted in squirting 200 µl of a 10% sucrose solution (with a syringe) on the mouse's snout that provokes in the mice a grooming behaviour.

Immediately after the squirting of the solution, mice were placed individually at the home cage and the behaviour was recorded for 5 minutes. The grooming time was manually measured using the recorded videos. Time of grooming is the sum of the paw and nose grooming, face grooming head grooming and all the rest of body parts licking (Kalueff *et al*., 2016).

7.9. Data and statistical analysis

Data were represented as mean ± S.E.M. The statistical analyses were performed using Student's *t*-test (to compare the effect of the genotype), two-way ANOVA or three-way ANOVA (with treatment, genotype, and model as variables), followed by Newman-Keuls *post-hoc* test where appropriate. The type of statistical analysis is indicated in the results section and in the legends of figures. The level of significance was set at $p < 0.05$. Graphs editing and statistical analyses were performed using the GraphPad Prism 6 version 6.01 for Windows (GraphPad Software, Inc., La Jolla, USA) and IBM SPSS statistics 24 (SPSS Inc., Chicago, USA).

8. (+)-8-OH-DPAT-INDUCED HYPOTHERMIA

The protocol was adapted from one previously reported (Zazpe *et al*., 2006). The experiments were carried out between 10:00 am and 14:00 pm. The body temperature was evaluated for a period of 15 s, or until a stable reading was obtained, by inserting a thermoelectric probe into the rectum. Initially, three measurements were made at 20 min intervals considering the average of the last two determinations as the basal temperature value. Then, (+)-8-OH-DPAT (1 mg/kg) was injected intraperitoneally and the body temperature was evaluated at 20 min and 40 min.

The hypothermic effect of (+)-8-OH-DPAT or vehicle was expressed as changes of temperature (ºC) (mean ± S.E.M.) respect to their basal values. Three-way ANOVA analysis followed by Newman-Keuls *post-hoc* test were performed. The type of statistical analysis is indicated in the results section and in the legends of figures. The level of significance was set at $p < 0.05$. Graphs editing and statistical analyses

were performed using the GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, USA) and IBM SPSS statistics 24 (SPSS Inc., Chicago, USA).

9. AUTORADIOGRAPHIC TECHNIQUES

9.1. Tissue preparation

Twenty-four hours after the last test, mice were sacrificed and their brains were rapidly removed and frozen immediately on dry ice and then stored at -80°C until sectioning. Coronal brain 14 μm thick sections were cut at -20°C using a microtome cryostat (Microm GmbH, Germany) and thaw-mounted in microscope slices SuperFrost® plus (Menzel-Gläser, Germany) and stored at -20°C until used for [^{35}S]GTPγS binding assays or at -80ºC in the case of *in situ* hybridization. Coronal brain sections were selected according to Franklin and Paxinos atlas (Bregma coordinates (in mm): 2.58 to 2.10 ([^{35}S]GTPγS and *in situ*), 1.10 to 0.86 (*in situ*), -2.06 to -2.54 ([^{35}S]GTPγS and *in situ*), -3.16 to -3.4 (*in situ*), -4.36 to -4.60 ([^{35}S]GTPγS), (Franklin and Paxinos, 2007).

9.2. [^{35}S]GTPγS autoradiography mediated by the stimulation of 5-HT$_{1A}$ receptor

Labelling of brain sections with [^{35}S]GTPγS was carried out as previously described (Castro *et al.,* 2008) in order to evaluate the functionality of 5-HT$_{1A}$ receptor, using the selective agonist 8-OH-DPAT. Slide-mounted sections were pre-incubated for 30 min at room temperature in a buffer containing 50 mM Tris-HCl, 0.2 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dl-dithiothreitol and 2 mM GDP at pH = 7.7. Slides were subsequently incubated, for 2 h, in the same buffer containing adenosine deaminase (3 mU/ml) with [^{35}S]GTPγS (0.04 nM) and consecutive sections were co-incubated with (±)-8-OH-DPAT (10 μM). The non- specific binding was determined in the presence of 10 μM guanosine-5-O-(3-thio) triphosphate (GTPγS). After the incubation, the sections were washed twice for 15 min in cold 50 mM Tris-HCl buffer (pH = 7.4) at 4°C, rinsed in distilled cold water and then dried under a cold air stream. Sections were exposed to film BioMax MR (Carestream Health, New York, USA) together with ^{14}C microscales at 4ºC for 2

days. Afterwards, films were immersed in the developer solution (Kodak, New York, USA) for 5 minutes at room temperature, and then were also immersed in the fixing solution (Kodak, New York, USA) for 5 minutes at room temperature. Finally, the films were washed in water to remove trace of developer and fixing solutions. At the end, the films were scanned and these images were used for further analysis.

9.3. *In situ* hybridization

Coronal brain 14 µm thick sections were cut at -20°C using a microtome cryostat and thaw-mounted in slices and stored at -80°C until the pretreatment of the slices. The *in situ* protocol was taken from Castro *et al*., (2003b). The brain sections were pretreated before starting the *in situ* hybridization. First, tissues were fixed with 4% paraformaldehyde in PBS for 5 minutes and then they were rinsed twice with a solution of PBS. Sections were acetylated with a solution of 0.25% acetic anhydride in 0.1 M triethanolamine buffer for 10 min. Then, brain sections were dehydrated with a graded ethanol wash (70%, 80%, 95% and 100%), were immersed in chloroform for 10 min, and were rehydrated with 100% and 95% ethanol. Finally, sections were air-dried and stored at -20°C prior to use.

Oligonucleotide complementary to BDNF mRNA 5'-GGTCTCGTAGAA ATATTGGTTCAGTTGGCCTTTTGATACCGGGAC-3' (Vaidya *et al*., 2001) *Arc* mRNA 5'-GCAGCTTCAGGAGAAGAGAGGATGGTGCTGGTGCTGG-3' (Kelly *et al*., 2008) and TrkB mRNA 5'-CCTTTCATGCCAA ACTTGGAATGTCTCGCCAACTTG-3' (Madhav *et al*., 2001) were 3´end-labelled with [^{35}S]dATP using terminal deoxynucleotide transferase (TdT). To labelling the probe, 3 pmoles of the specific oligonucleotide was mixed with 1.6 pmoles of [^{35}S]dATP (2' deoxyadenosine 5'-(α- thio) triphosphate) with an specific activity of 1250 Ci/mmol, 15 U of TdT, 2.5 µl TDT buffer and DEPC water. This mixture was incubated for 1 hour at 37ºC, and then the reaction was stopped with 87.5 µl of DEPC water. To remove the non-incorporated nucleotides the reaction mix with the probe was purified with Sephadex chromatography micro column illustra ProbeQuant G-50 (GE Healthcare life science, UK) following the kit instructions. After the purification, labelled probe (250000 c.p.m./slide) was mixed with hybridization buffer (50% deionized formamide, 4x standard saline citrate

(SSC), 10 mM sodium phosphate pH = 7.0, 1 mM sodium pyrophosphate, 10% dextran sulphate, 5x Denhardt's solution, 200 µg/ml salmon sperm DNA, 100 µg/ml poly A, 0.12 mg/ml heparin and 20 mM dithiothreitol). After the homogenisation, 200 µl of the mixture was evenly scattered for every slide, and they were coated with cover glasses (Menzel-Gläser, Germany). After incubation at 42°C for 16 hours in a humidified chamber, slides were washed to remove excess probe and reduce unspecific binding. Slides were washed at 50°C in 2x SSC buffer with 1M DTT twice for 30 minutes followed by three washes of 5 minutes at room temperature with 1x SSC, 0.1x SSC, and ethanol 80% consecutively. Finally, slides were washed in ethanol 96% for 1 minute at room temperature. Sections were air-dried and exposed to film BioMax MR together with ^{14}C microscales at -20°C for 2-4 weeks depending on the abundance of mRNA in the tissue. The control of specificity was done with the probe without labelling (at a concentration 1000 times higher). The films were processed following the same protocol described above, and they were scanned to generate a digital image to further analysis.

9.4. Quantification of the autoradiograms, data and statistical analysis

The autoradiograms generated were analysed and quantified using a computerized image analysis Scion Image software (Scion Corporation, Frederick, USA). This software allows the measurement of the optical density in brain regions. In both, [^{35}S]GTPγS autoradiography of the 5-HT_{1A} receptor and *in situ* hybridization, radioactive ^{14}C microscales were used to construct a calibration curve that correlates the radioactivity levels (measured as nCi/g of estimated tissue equivalent) and the optical density. The data of [^{35}S]GTPγS autoradiography of 5-HT_{1A} receptor were represented as % of stimulation of [^{35}S]GTPγS binding induced by 8-OH-DPAT. This parameter was calculated as a percentage of 8-OH-DPAT-stimulated binding respect the specific basal binding. In the case of the *in situ* hybridization, the data were expressed in nCi/g of estimated tissue equivalent.

Data were represented as mean ± S.E.M. The statistical analyses were performed using Student's *t*-test (to compare the effect of the genotype) or two-way ANOVA and three-way ANOVA (with treatment, genotype, and model as variables),

followed by Newman-Keuls *post-hoc* test where appropriate. The type of statistical analysis is indicated in the results section and in the legends of figures. The level of significance was set at $p < 0.05$. Graphs editing and statistical analyses were performed using the GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, USA) and IBM SPSS statistics 24 (SPSS Inc., Chicago, USA).

10. IMMUNOHISTOCHEMISTRY OF BrdU

10.1. Tissue preparation

A day before the sacrifice, four injections of BrdU separated by two hours were administered intraperitoneally. Twenty-four hours after the last BrdU injection, mice were deeply anaesthetized with pentobarbital sodium and were transcardially perfused with saline followed by 4% cold paraformaldehyde in PBS. The brains were removed, postfixed overnight at 4ºC and transferred to 30% sucrose in PBS at 4ºC. Serial coronal sections (40 µm) of the brains were obtained through the entire hippocampus on a microtome cryostat (Microm GmbH, Germany) and stored at -20ºC in a cryoprotectant solution (25% glycerol, 25% ethylene glycol in PBS, pH = 7.4).

10.2. Immunohistochemistry protocol

BrdU staining was performed as previously described (Mostany *et al*., 2008). Free-floating coronal sections were incubated for 2 h in 50% formamide/2x SSC at 65°C, followed by incubation in 2N HCl for 30 min. Then, sections were incubated for 10 min in 0.1M borate buffer. After washing in PBS, sections were incubated in 1% H_2O_2 in PBS for 30 min to inactive endogenous peroxidase activity. After several rinses in PBS, sections were incubated in PBS/0.2% Triton X-100/5% goat serum (PBS-TS) for 30 min and then incubated with monoclonal mouse anti-BrdU antibody (1:600; ref.: 11170376001 Roche Diagnostics, Barcelona, Spain) overnight at 4°C. After several rinses in PBS-TS, sections were incubated for 2 h with biotinylated goat anti-mouse Fab Fragment IgG secondary antibody (1:200; ref.: 115-066-006 Jackson ImmunoResearch Laboratories, Inc., US-PA), followed by amplification with avidin-biotin complex (Vector Laboratories).

10.3. Data and statistical analysis

For quantification of $BrdU^+$ cells, one every sixth section throughout the hippocampus was processed and counted under a light microscope (Carl Zeiss Axioskop 2 Plus) at 40x and 100x magnification. The total number of $BrdU^+$ cells *per* section were determined and multiplied by 6 to obtain the total number of $BrdU^+$ cells *per* hippocampus. Data were represented as percentage of the mean of WT group (considering the mean of this group as 100%). Data were represented as mean ± S.E.M. The statistical analyses were performed using Student's *t*-test (to compare the effect of the genotype). The type of statistical analysis is indicated in the results section and in the legends of figures. The level of significance was set at $p < 0.05$. Graphs editing and statistical analyses were performed using the GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, USA).

11. WESTERN BLOT

11.1. Tissue preparation

The mice were sacrificed and their brains were removed. The hippocampi were dissected on ice and immediately stored at -80ºC. The total hippocampal protein extraction protocol was adapted from Chomczynski (1993). For each hippocampus, 250 µl of TRIzol® reagent a solution of phenol and guanidine isothiocyanate was added. The samples were homogenised with the aid of an electric homogeniser (pellet pestles cordless motor, ref: Z359971, Sigma-Aldrich Química SL, Madrid, Spain) and were incubated for 5 minutes at room temperature. Then, 50 µl of chloroform were added and the tubes were manually shaken for 15 seconds, and incubated for 2 minutes at room temperature. Afterwards, the samples were centrifuged at 11400 r.p.m. for 15 minutes at 4ºC to separate the DNA, RNA and proteins. After centrifugation, the mixture was separated into three phases, a clear upper aqueous layer containing RNA which was discarded, an interphase containing DNA pellet and a red lower organic layer containing proteins and DNA. Then, 1 mL of 100% ethanol was added to each sample, and they were shaken manually for about 15 seconds. Afterwards, samples were incubated at room temperature for 2-3 minutes and were centrifuged at 3000 r.p.m. for 5 minutes at 4ºC. After the

centrifugation, DNA was precipitated and supernatants were transferred into clean Eppendorf tube to continue with protein extraction. Next, 1mL of isopropanol was added and the samples were incubated at room temperature for 10 minutes followed by a centrifugation at 11400 r.p.m. for 10 minutes at 4ºC. The supernatant was discarded and 500 µl of washing solution (consisting of 0.3 M of guanidine hydrochloride in 95% ethanol) were added to the protein pellets. The mixture was homogenised and incubated for 20 minutes at room temperature and centrifuged at 11400 r.p.m. for 5 minutes at 4ºC. This washing step was repeated two times more. After the washes, 1 mL of 100% ethanol was added, and the samples were vigorously vortexed and incubated for 20 minutes at room temperature. The samples were centrifuged again at 9000 r.p.m. for 5 minutes at 4ºC, and the supernatants were discarded. The protein pellets obtained were air-dried at room temperature for 10 minutes and resuspended in a 4 M solution of urea and 1% SDS and incubated overnight at 50ºC. Then, the samples were centrifuged at 10000 x g for 10 minutes at 4ºC and the supernatants were stored at -20ºC until use. After the extraction, an aliquot of each extract was used to measure the protein concentration, and the amount of protein was determined by a colorimetric assay using the Bio RAD DC kit (as described in section 5.3.). Protein extracts were dissolved in a 1:1 ratio with Laemmli buffer (Bio-Rad, California, USA), which contains β-mercaptoethanol at a 1:20 dilution. Then, samples were incubated for 5 minutes at 100ºC and were placed immediately in ice. Samples were centrifuged at 3000 r.p.m. for 5 minutes and the supernatants were collected and stored at -20ºC.

11.2. Western blot protocol

First, the polyacrylamide gels were prepared using distilled water, 8.5% of acrylamide-BIS solution, 4% of separation buffer (1.5 M of Tris and 0.4% of SDS, pH = 8.8), 10% ammonium persulfate (APS) and TEMED. The mixture was poured between the two crystals of the Mini-PROTEAN Tetra Cell system (Bio rad, Hercules, USA). The separation gel was allowed to polymerize and the stacking gel was placed on top of the separation gel. The composition of the stacking gel was: distilled water, 4% acrylamide-bis solution, 4X stacking gel buffer (0.5 M Tris-HCl and 0.4% of SDS, pH = 6.8), 10% APS, Phenol red and TEMED.

The gels were assembled in pairs and placed in an electrophoresis cuvette which was filled with 1 x migration buffer (25 mM of Tris, 190 mM of glycine and 0.1% SDS, pH = 8.3). Combs were removed and the wells were cleaned. Then, 30 µg of protein for each sample was loaded with a Hamilton syringe (Hamilton, Nevada, USA). After loading all the samples, the electrophoresis was started. The electrophoresis conditions were 100 volts for 15 minutes followed by 50 minutes at 160 volts. At the end of the migration, gels, nitrocellulose membranes (GE Healthcare Europe GmbH, Munich, Germany) and Whatman papers were immersed in the transfer buffer (25 mM of Tris, 190 mM of glycine 0.1 % of SDS and 20 % of methanol) for 30 minutes. Proteins were wet-transferred for 90 minutes at 100V onto nitrocellulose membranes. Subsequently, non-specific binding of the antibodies to the membranes was blocked with a solution that contains TBS-T buffer (150 mM NaCl, 50 mM Tris-HCl and 0.05% Tween 20) with 3% (W/V) nonfat dry milk, 1mM of sodium vanadate and 1 mM of sodium phosphate (as a phosphatase inhibitors). Then, membranes were incubated with the primary antibodies overnight. The following primary antibodies were used: Rabbit Anti-p-mTOR (1:250, Cell signaling Technology, Inc., Danvers, USA number of reference # 4517), mouse anti-mTOR (1:1000, Cell signaling Technology, Inc., Danvers, USA number of reference # 2971), mouse anti-β-catenin (1:1000, Santa Cruz Biotechnology, Dallas, USA, number of reference sc-7963), rabbit anti-p-β-catenin (1:200, Santa Cruz Biotechnology, Dallas, USA, number of reference sc-16743-R), and mouse anti-tubulin (1: 20,000 Sigma-Aldrich, San Luis, USA, number of reference T-8660) as a housekeeping. The next morning, three washes of 15 minutes were done in TBS-T. Then, the membranes were incubated with secondary anti-rabbit and anti-mouse antibodies conjugated to a fluorophore that emits and absorbs in the near infrared wavelength (1:15000, LI-COR Bioscience, Lincoln, USA, numbers of reference P/N 925-68072 and P/N 925-32213). After the incubation, three washes in TBS-T were made and the membranes were developed using the Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, USA).

11.3. Data and statistical analysis

Western blot analysis was performed by using Image Studio™ Lite program (LI-COR Bioscience, Lincoln, USA) and the densitometric values were normalized

with respect to the values obtained with the housekeeping protein tubulin. Data are expressed as percentage of the mean of the values of the respective WT group (considering the mean of this group as 100%). Data are represented as mean ± S.E.M. The statistical analyses were performed using Student's *t*-test (to compare the effect of the genotype) or two-way ANOVA and three-way ANOVA (with treatment, genotype, and model as variables), followed by Newman-Keuls *post-hoc* test where appropriate. The type of statistical analysis is indicated in the results section and in the legends of figures. The level of significance was set at $p < 0.05$. Graphs editing and statistical analyses were performed using the GraphPad Prism 6 version 6.01 for Windows (GraphPad Software, Inc., La Jolla, USA). 6.0 (La Jolla California USA) and IBM SPSS statistics 24 (SPSS Inc., Chicago, IL).

"*Lo importante en la ciencia no es tanto obtener nuevos datos, sino descubrir nuevas formas de pensar sobre ellos.*"

William Lawrence Bragg

IV. Results

1. CHARACTERIZATION OF 5-HT$_4$ KO MICE

1.1. Lack of 5-HT$_4$ receptor stimulated adenylate activity in 5-HT$_4$ KO mice

First, and before carrying out the experimental procedures, the functionality of 5-HT$_4$ receptors was evaluated in KO mice, in order to confirm the absence of functionality of the receptor in these animals. For this purpose, as 5-HT$_4$ receptors are positively coupled with adenylate cyclase, the level of the second messenger cAMP induced by the stimulation of the receptor with a specific agonist was evaluated. cAMP basal values in striatal membranes were similar in both genotypes (17.1 ± 1.7 *vs* 19.0 ± 1.9 pmol/min/mg protein for WT and 5-HT$_4$ KO mice, respectively). The 5-HT$_4$ agonist zacopride did not produce any change in 5-HT$_4$ receptor-induced cAMP accumulation in 5-HT$_4$ KO mice, (98.8 ± 15.1% zacopride-induced stimulation *vs* 100.0 ± 8.1% basal values) compared to the increase observed in WT mice (175.7 ± 26.7% zacopride-induced cAMP accumulation *vs* 100.0 ± 2.9% basal values; $p < 0.01$). These data confirm the lack of 5-HT$_4$ receptors functionality in these 5-HT$_4$ KO mice (Fig. 12).

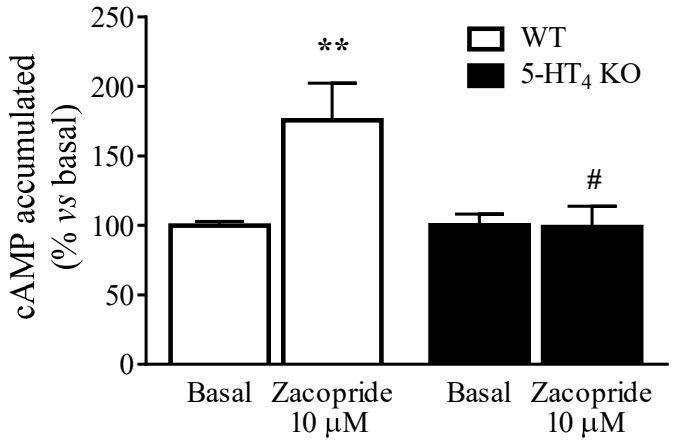


Figure 12. cAMP accumulation induced by zacopride (10 µM) in the striatum. Two-way ANOVA analysis revealed a main effect of genotype ($F_{(1,23)}$: 6.8, $p < 0.05$), zacopride ($F_{(1,23)}$: 6.4, $p < 0.05$) and a genotype x zacopride interaction ($F_{(1,23)}$: 7.1, $p < 0.05$). $^{**}p < 0.01$ *vs* WT-basal and $^{\#}p < 0.05$ *vs* WT-zacopride (Newman-Keuls *post hoc* test). Data are mean ± SEM considering 100% of the basal binding value for each condition. n = 5-7 mice per group and duplicated samples.

1.2. Behavioural phenotype of 5-HT$_4$ KO mice

1.2.1. Anxiety-like behaviour

Initially, we evaluated whether the genetic deletion of 5-HT$_4$ receptor might influence anxiety-like behaviour of mice by using ethological tests (approach-avoidance conflict), namely open field and light/dark box tests. In the open field test (OF), 5-HT$_4$ KO mice presented lower central activity as evidenced by a reduction in the central time (46.7 ± 3.0 s) compared with WT counterparts (60.4 ± 5.5 s, $p < 0.05$) (Fig. 13A), with a similar number of entries in the central area (WT: 28.4 ± 1.5, KO: 24.0 ± 1.9) (Fig. 13B). This anxious phenotype, it was not associated with altered locomotion because mice of both genotypes travelled a similar total distance (WT: 20.7 ± 1.5 m, KO: 21.4 ± 0.9 m) (Fig. 13C). In the light/dark box, no significant differences were found between mice of both genotypes in the time spent in the light zone (WT: 90.8 ± 11.2 s, KO: 80.5 ± 9.0 s) (Fig. 13D). Moreover, in the marble burying test (an active avoidance test) 5-HT$_4$ KO mice presented a reduction of the number of marbles buried respect to WT mice (WT: 7.1 ± 1.0, KO: 4.3 ± 0.8, $p < 0.05$) (Fig. 13E). Finally, mice were assessed in the novelty suppressed feeding (NSF) test as a hypohenophagia-type behavioural paradigm. 5-HT$_4$ KO mice did not show significant changes in the latency to feed in this test (WT: 203.9 ± 35.6 s; KO: 219.7 ± 29.5 s) (Fig. 13F). In addition, mice of both genotypes consumed a similar amount of food (g/5-min session) when returned to the home-cage immediately after performing the NSF test (WT: 0.15 ± 0.01 g, KO: 0.16 ± 0.01 g) (Fig. 13G).

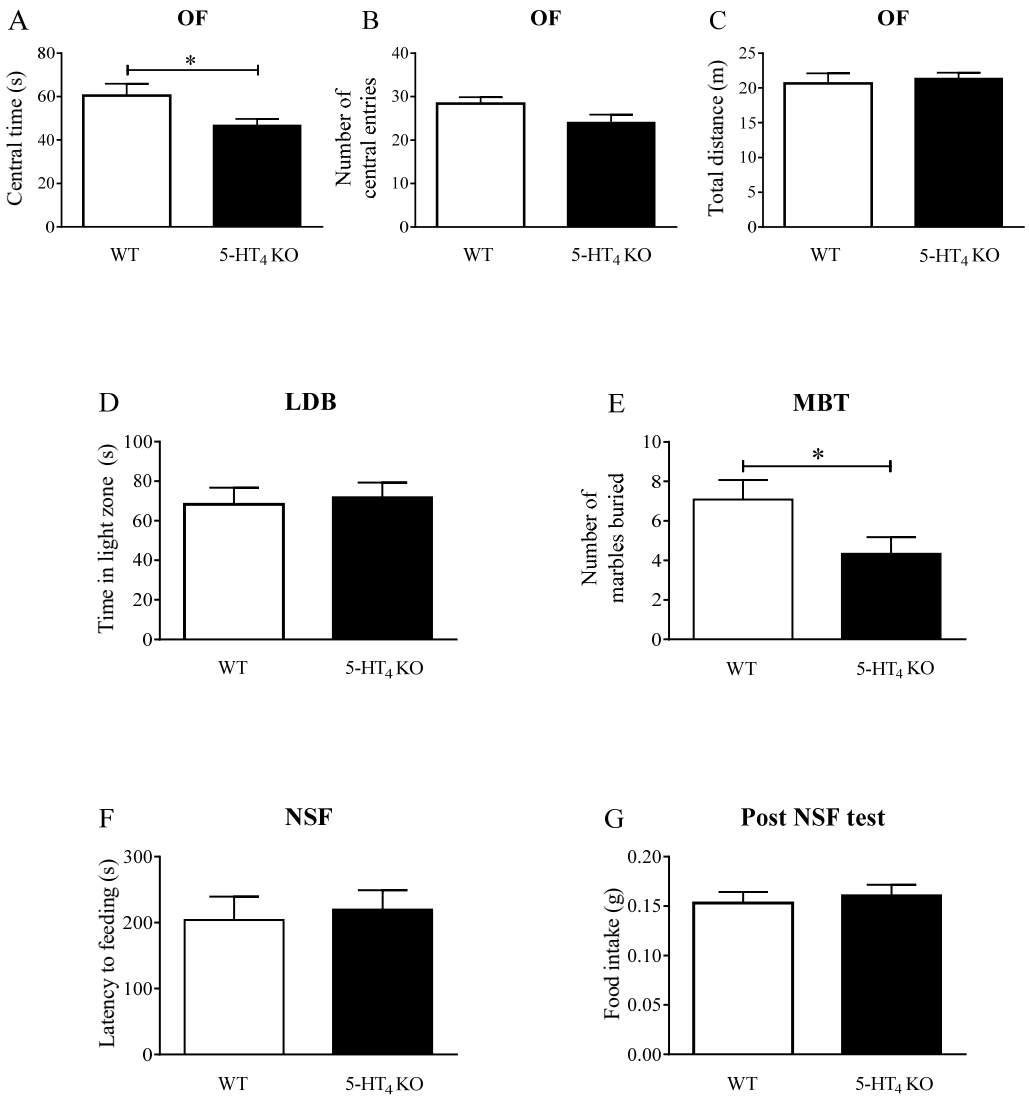


Figure 13. Behavioural responses in different anxiety-related paradigms. In the open field test, central time (**A),** number of entries in the central zone (**B)** and total distance travelled (**C)** were measured. Time in the light zone of the light/dark box (**D**), number of marbles buried in MBT (**E**), latency to feeding in the NSF (**F**), and food intake after NSF (**G**). Data are mean ± SEM of n = 13-18 mice per group. *$p < 0.05$ (Student t-test, unpaired data).

1.2.2. Depression-related behaviour

Different tests were carried out to evaluate whether these animals exhibited different emotional responses linked to a depressive-like trait. Anhedonia (lack of interest in rewarding stimuli) was modelled using the sucrose intake paradigm. 5-HT_4 KO animals showed a lower sucrose intake than WT counterparts (KO: 1.5 ± 0.1 ml *vs* WT: 2.0 ± 0.1 ml, $p < 0.001$) (Fig. 14A). Depression-associated cognitive deficits related to hippocampal functioning were investigated using the nesting test. An impaired performance was observed in 5-HT_4 KO mice (nesting score of KO: 3.8 ± 0.3 *vs* WT: 4.8 ± 0.1, $p < 0.01$) (Fig. 14B). Additionally, both genotypes exhibited a similar time of grooming in the splash test (WT: 46.6 ± 13.1 s *vs* KO: 44.3 ± 7.4 s) (Fig. 14C). Finally, behavioural despair was assessed in the FST. In this paradigm, mice of both genotypes exhibited similar immobility (WT: 206.4 ± 6.2 s *vs* KO: 204.7 ± 9.1 s) (Fig. 14D), swimming (WT: 30.3 ± 5.4 s *vs* KO: 33.1 ± 8.5 s) (Fig. 14E) and climbing (WT: 3.4 ± 1.1s *vs* KO: 2.1 ± 1.1 s) (Fig. 14F) scores.

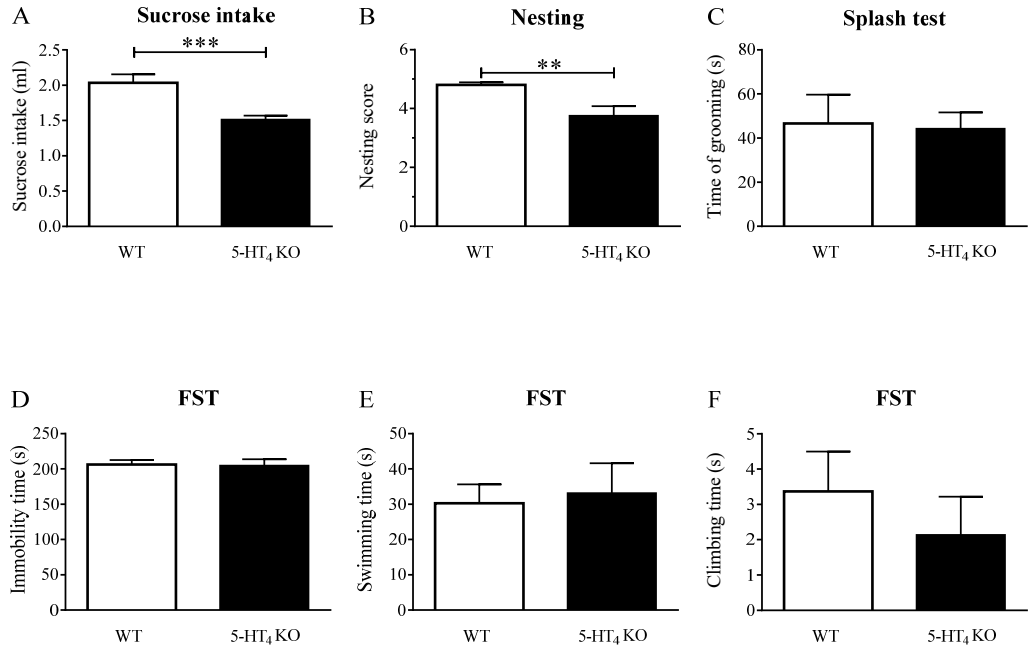


Figure 14. Behaviour performance in different depression-related paradigms. 5-HT_4 KO mice exhibited reduced sucrose intake (**A**) and reduced nesting behaviour (**B**) compared with WT mice. No differences were observed between mice of both genotypes in the time of grooming in the splash test (**C**) and neither all the FST parameters [immobility (**D**), swimming (**E**) and climbing (**F**)]. Data are mean ± SEM of n = 13-20 mice per group. $^{**}p < 0.01$ and $^{***}p < 0.001$ (Student t-test, unpaired data).

1.3. 5-HT$_{1A}$ receptor sensitivity in 5-HT$_4$ KO mice

1.3.1. *In vitro* evaluation of 5-HT$_{1A}$ receptor functionality

Due to the relevance of 5-HT$_{1A}$ receptor in the depression and in the mechanism of action of antidepressant drugs, the 5-HT$_{1A}$ receptor functionality was evaluated *in vitro* by measuring (±)-8-OH-DPAT stimulated [^{35}S]GTPγS binding in brain sections.

First, the specific basal [^{35}S]GTPγS binding on different brain areas in WT and 5-HT$_4$ KO mice was compared. In both genotypes, the dorsal raphe nucleus (DRN) was the region exhibiting the highest value whereas the rest of brain areas showed similar values (~ 200 nCi/g tissue in WT and ~ 240 nCi/g tissue in KO mice). When comparing both genotypes, an increase in basal [^{35}S]GTPγS binding values was detected in the brain of 5-HT$_4$ KO mice in some serotonergic brain nuclei such as the DRN (+21% *vs* WT, $p < 0.05$) and median raphe nucleus (MRN; +21% *vs* WT, $p = 0.074$). Increases in specific basal [^{35}S]GTPγS binding was also observed in some limbic structures such as the medial prefrontal cortex (+40% *vs* WT, $p < 0.01$), frontal cortex (+36% *vs* WT, $p < 0.01$), CA1 oriens (+17% *vs* WT, $p < 0.05$) and CA3 (+15% *vs* WT, $p = 0.053$) hippocampal fields. No differences were detected in the other areas analysed (Table 3 and Fig. 15).

The stimulation of specific [^{35}S]GTPγS binding induced by (±)-8-OH-DPAT in control conditions (WT mice) is shown in table 4. In a range of order, a different degree of stimulation was observed among the brain areas: a) high values (+60-80% of specific basal binding) in the DRN, CA1 field of hippocampus, and entorhinal cortex; b) intermediate values (+20-40% of specific basal binding) in MRN, mPFrCx, FrCx and, c) low values (below +20% of specific basal binding) in CA3 and DG in the hippocampus. 5-HT$_4$ KO mice displayed a similar level of (±)-8-OH-DPAT-induced stimulation than WT mice with the exception of the DRN (Table 4 and Fig. 15). Indeed, in this area the stimulation of specific [^{35}S]GTPγS binding induced by the 5-HT$_{1A}$ agonist was significantly lower than in WT counterparts (+39.0% in KO mice *vs* +69.5% in WT mice, $p < 0.01$). In contrast, no significant differences were detected in other areas (Table 4).

Table 3. Specific basal [^{35}S]GTPγS binding values (nCi/g tissue). DRN: dorsal raphe nucleus, MRN: median raphe nucleus, mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Ent Cx: entorhinal cortex, CA1 oriens: CA1 oriens hippocampal field, CA1 rad; CA1 radiatum hippocampal field, CA3: CA3 hippocampal field, DG: dentate gyrus. Data are mean ± SEM of n = 6-9 mice per group. *p < 0.05 and **p < 0.01 (Student's t-test, unpaired data).

	WT	5-HT$_4$ KO
Brainstem areas		
DRN	299.9 ± 7.5	**362.2 ± 22.8***
MRN	194.2 ± 9.3	236.4 ± 22.8
Cortical areas		
mPFrCx	204.8 ± 17.3	**286.6 ± 12.8****
FrCx	183.3 ± 15.0	**249.6 ± 16.5****
Ent Cx	215.7 ± 12.4	245.8 ± 17.2
Hippocampal areas		
CA1 oriens	186.2 ± 9.8	**218.2 ± 12.2***
CA1 rad	227.8 ± 7.1	244.0 ± 13.1
CA3	226.9 ± 15.2	261.9 ± 8.8
DG	183.7 ± 4.9	198.8 ± 8.6

Table 4. Stimulation of specifc [^{35}S]GTPγS binding induced by (±)-8-OH-DPAT (% of basal values). DRN: dorsal raphe nucleus, MRN: median raphe nucleus, mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Ent Cx: entorhinal cortex, CA1 oriens: CA1 oriens hippocampal field, CA1 rad; CA1 radiatum hippocampal field, CA3: CA3 hippocampal field, DG: dentate gyrus. Data are mean ± SEM of n = 6-9 mice per group. *p < 0.05 and **p < 0.01 (Student's t-test, unpaired data).

	WT	5-HT$_4$ KO
Brainstem areas		
DRN	69.5 ± 6.9	**39.0 ± 6.1****
MRN	38.1 ± 9.9	32.2 ± 13.3
Cortical areas		
mPFrCx	36.5 ± 6.7	15.8 ± 9.4
FrCx	24.8 ± 4.6	17.3 ± 8.3
Ent Cx	69.4 ± 14.2	74.2 ± 19.8
Hippocampal areas		
CA1 oriens	70.5 ± 14.2	76.3 ± 15.4
CA1 rad	85.3 ± 14.3	100.5 ± 15.0
CA3	5.8 ± 7.2	14.0 ± 4.3
DG	18.8 ± 7.3	13.8 ± 5.3

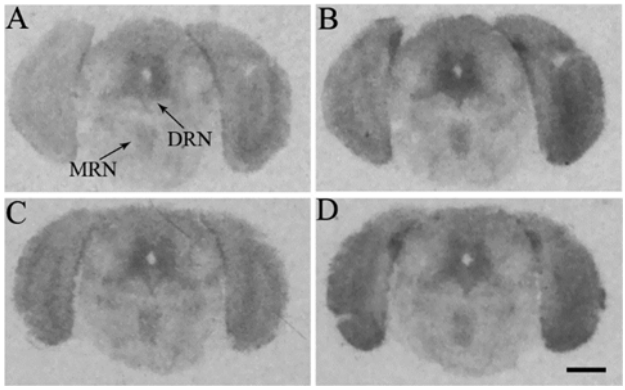


Figure 15. Representative autoradiograms of [^{35}S]GTPγS binding. in coronal midbrain sections. Upper: WT mice, basal (**A**) and stimulated (**A'**) binding. Lower: 5-HT$_4$ KO mice, basal (**B**) and stimulated binding (**B'**). DRN: dorsal raphe nucleus, MRN: median raphe nucleus. Scale bar = 1 mm.

1.3.2. *In vivo* evaluation of 5-HT$_{1A}$ autoreceptor functionality

Since we observed a reduction in G-protein signalling of DRN 5-HT$_{1A}$ receptors in 5-HT$_4$ KO mice, we also assessed their functionality *in vivo* by measuring (+)-8-OH-DPAT-induced hypothermia, a pharmacological effect critically dependent on 5-HT$_{1A}$ autoreceptors. The maximal decrease in body temperature was obtained 20 minutes after (+)-8-OH-DPAT administration (WT: -2.7 ± 0.3ºC) and was maintained after 40 minutes. Our results indicate that 5-HT$_4$ KO mice exhibited a similar basal temperature (WT: 36.9 ± 0.2ºC *vs* KO: 36.9 ± 0.2ºC) as well as a similar hypothermic effect in response to the 5-HT$_{1A}$ agonist (KO: -2.7 ± 0.3ºC) than WT counterparts (Fig. 16)

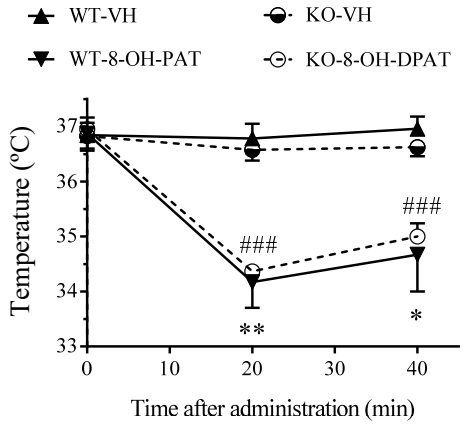


Figure 16. Effect of (+)-8-OH-DPAT induced hypothermia in mice. Time course of the temperature in vehicle and (+)-8-OH-DPAT (1 mg/kg) administration in mice. Note that no significant differences were found in the hypothermic effect of (+)-8-OH-DPAT between genotypes. Three-way ANOVA analyses revealed a significant interaction effect of time x treatment ($F_{(2,17)}$: 43.2, $p < 0.001$) and also a significant main effect of the time ($F_{(2,17)}$: 55.4, $p < 0.001$). Data are mean ± SEM of n = 5-6 mice per group. *$p < 0.05$, and **$p < 0.001$ *vs* WT-VH, $^{###}p < 0.001$ *vs* KO-VH (Newman-Keuls *post hoc* test).

1.4. Altered BDNF, TrkB and *Arc* mRNA expression in 5-HT$_4$ KO mice

1.4.1. Influence of genotype in BDNF and TrkB mRNA expression

Given the relevance that BDNF and its cognate receptor TrkB have on the depressive disorder, the mRNA levels of both proteins were evaluated in KO mice. The distribution of BDNF mRNA expression was similar to that reported in previous studies in rats (Pascual-Brazo *et al.,* 2012), with highest levels in the hippocampus (ranged 12.5 - 39.1 nCi/g tissue) and cortical areas (ranged 5.6 - 19.7 nCi/g tissue), and lower levels in other regions including striatum or thalamus (data not shown) (Figs. 17A and C). However, high levels of TrkB mRNA were found across the brain (Figs. 17B and C). The non-specificity of BDNF and TrkB mRNA labelling was determined by the displacement with excess of unlabeled oligonucleotide and represented less than 5% of the total binding.

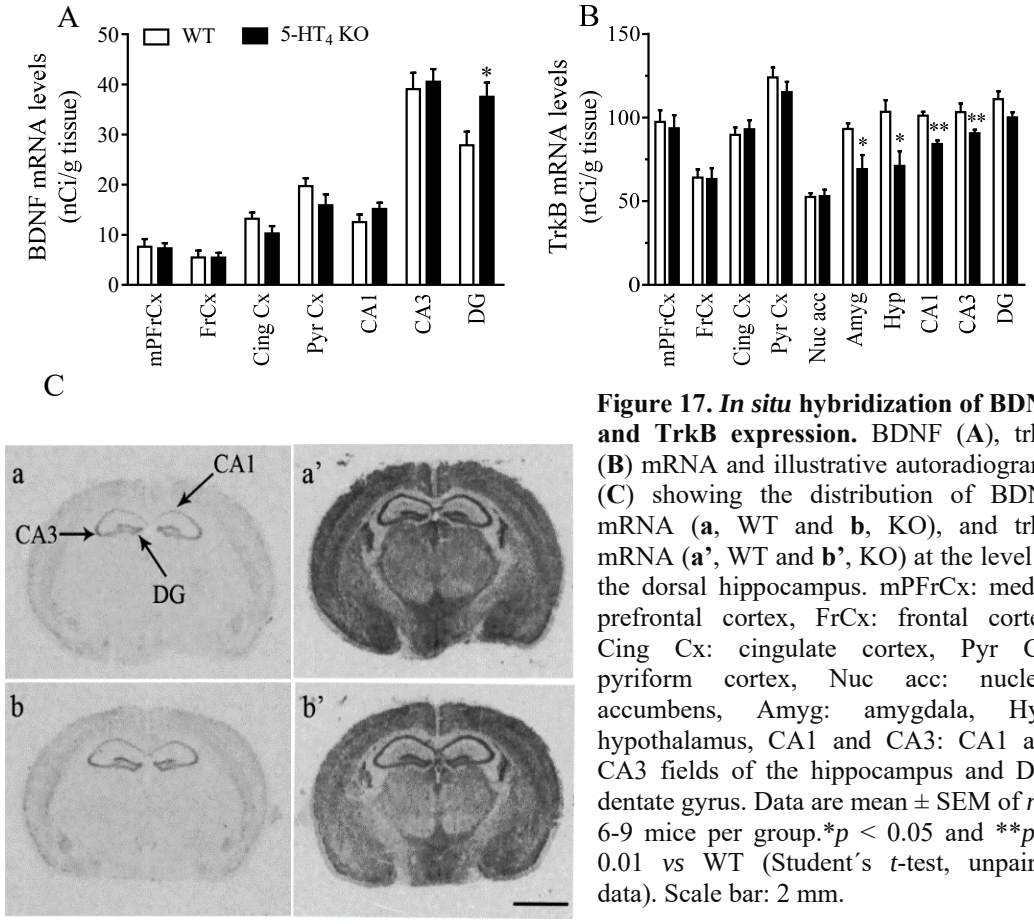


Figure 17. *In situ* hybridization of BDNF and TrkB expression. BDNF (**A**), trkB (**B**) mRNA and illustrative autoradiograms (**C**) showing the distribution of BDNF mRNA (**a**, WT and **b**, KO), and trkB mRNA (**a'**, WT and **b'**, KO) at the level of the dorsal hippocampus. mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, Nuc acc: nucleus accumbens, Amyg: amygdala, Hyp: hypothalamus, CA1 and CA3: CA1 and CA3 fields of the hippocampus and DG: dentate gyrus. Data are mean ± SEM of n = 6-9 mice per group.*$p < 0.05$ and **$p < 0.01$ *vs* WT (Student´s *t*-test, unpaired data). Scale bar: 2 mm.

Regarding the effect of genotype, an altered BDNF and TrkB mRNA expression was observed in 5-HT$_4$ KO mice when compared with their counterparts. Thus, 5-HT$_4$ KO mice showed higher levels of BDNF mRNA in the dentate gyrus (DG) of the hippocampus than WT mice (~35%; $p < 0.05$) (Figs. 17A and C), which was not associated with significant changes in the levels of trkB mRNA (Figs. 17B and C). Additionally, 5-HT$_4$ KO mice exhibited reduced levels of trkB mRNA in the other hippocampal fields (CA1 and CA3: ~15% *vs* WT, $p < 0.01$), in the hypothalamus and amygdala (~30% *vs* WT, $p < 0.05$) (Figs. 17B and C). No differences were detected in the levels of trkB and BDNF mRNA in the cortical areas between mice of both genotypes (Figs. 17A, B and C).

1.4.2. Influence of genotype in *Arc* mRNA expression

Regional distribution of the *Arc* mRNA was variable throughout the mice brain as previously reported in previous studies (Kelly *et al.*, 2008) (Fig. 18). *Arc* mRNA levels in WT mice in representative brain regions ranged from: 22.8 - 41.3 nCi/g tissue in hippocampus and 16.2 - 37.4 nCi/g tissue in cortical areas. The levels of mRNA encoding *Arc* were higher in the CA1 and CA3 hippocampal fields and the cingulate cortex in 5-HT$_4$ KO mice (~ 50%, $p < 0.05$) (Figs. 18A and B) compared with WT mice.

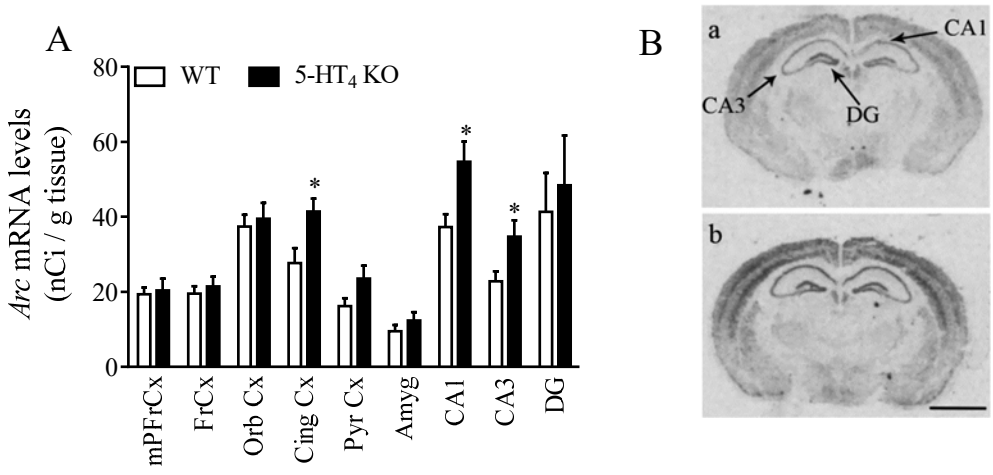


Figure 18. *In situ* hybridization of *Arc* mRNA. Levels of *Arc* mRNA (**A**) and autoradiograms depicting the distribution of *Arc* mRNA in coronal brain sections (**B**) from WT (**a**) and 5-HT$_4$ KO (**b**) mice, at the level of the dorsal hippocampus. mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, Amyg: amygdala, CA1 and CA3: CA1 and CA3 fields of the hippocampus and DG: dentate gyrus. Data are mean ± SEM of n = 6-9 mice per group.*$p < 0.05$ *vs* WT (Student´s *t*-test, unpaired data). Scale bar: 2 mm

1.5. Absence of impaired hippocampal proliferation in 5-HT$_4$ KO mice

5-HT$_4$ KO mice presented alterations in some neuroplasticity markers are known to be implicated in hippocampal proliferation, thus, an immunohistochemistry of BrdU$^+$ in the dentate gyrus was carried out. A similar number of BrdU immunolabelled cells was detected in both 5-HT$_4$ KO (1522.0 ± 149.3 BrdU$^+$ cells) and WT (1483.0 ± 109.3 BrdU$^+$ cells) mice (Figs. 19A and B).

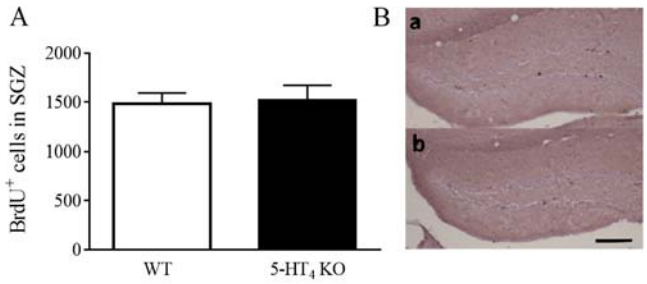


Figure 19. Immunhistoche-mistry of BrdU$^+$ in the dentate gyrus. Total BrdU$^+$ cells in the subgranular zone (SGZ) of the hippocampus **(A)** and, illustrations showing BrdU immunopositive cells in the DG (**B)** in WT **(a)** and 5-HT$_4$ KO (**b**) mice.Data are mean ± SEM *n* = 6-7 animals per group. Scale bar: 20 μm.

1.6. Immnunodetection of mTOR and β-catenin and their phosphorylated forms in the hippocampus

In the last decade, several studies demonstrate the implication of some neuroplasticity –related pathways in the neurobiology of depression and antidepressant effects. Among them, mTOR and β-catenin signalling are some of the most promising. Therefore, we analysed the levels of expression of mTOR and β-catenin and their phosphorylated forms in the hippocampus of WT and 5-HT$_4$ KO mice.

5-HT$_4$ KO mice presented a lower ratio of p-mTOR/mTOR expression (-38.3% *vs* WT, $p < 0.05$) (Fig. 20B) without changes in the absolute mTOR protein levels compared with WT mice (Fig. 20A). No differences were found between the two genotypes regarding either β-catenin protein levels (Fig. 20C) or p-β-catenin/β-catenin ratio (Fig. 20D).

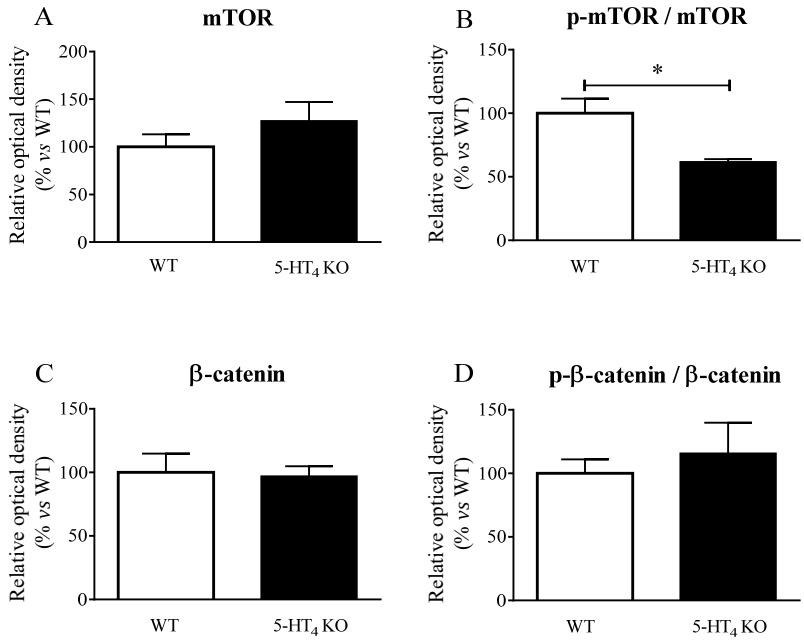


Figure 20. Protein levels of mTOR and β-catenin and their phosphorylated forms in the hippocampus. Protein levels of mTOR (**A**), p-mTOR/mTOR (**B**), β-catenin (**C**) and p-β-catenin/β-catenin ratio (**D**) in WT and KO mice. Data are mean ± SEM, n = 5-8 animals per group. *$p < 0.05$ *vs* WT (Student´s *t*-test, unpaired data).

2. CORTICOSTERONE MODEL OF DEPRESSION: EFFECT OF RS67,333 AND FLUOXETINE

In the previous results section, we have analysed the behavioural, molecular and neurochemical consequences of the loss of function of the 5-HT4 receptors. Afterwards, 5-HT4 receptor KO mice - and *wild type* counterparts- were subjected to chronic corticosterone administration. This is known to be an animal model of depression with a high construct validity since it mimics how a maladaptive-stress response affects the HPA axis functioning and leads to behavioural and neuroplastic changes related to depression and anxiety. Moreover, we have also evaluated and compared the effect of the chronic administration of fluoxetine and RS67,333 in this animal model. All these studies were intended to determine whether the lack of 5-HT4 receptors could account for a differential vulnerability to manifest anxiety-depression-like states under "pathological" conditions. Also, they could give

valuable information regarding the contribution of 5-HT_4 receptors to the pharmacological actions induced by chronic administration of RS67,333 and fluoxetine.

2.1. Behavioural studies

2.1.1. Chronic corticosterone model

After 4 weeks of corticosterone administration, 5-HT_4 KO mice and WT counterparts were tested in several behavioural paradigms in order to confirm the development of this "pathological" model and to compare the anxiety- and depressive-like responses in both genotypes. Our results confirmed that chronic corticosterone administration induced a quite similar anxious phenotype in both genotypes. For instance, a similar enhanced anxiety-like response following corticosterone administration was observed in both genotypes as evidenced by a decrease in OF central activity (i.e. time and entries) when compared with their respective counterparts (Figs. 21A and B), which was not due to changes in the locomotor activity (Fig. 21C). In good concordance, a similar corticosterone-induced increase in the latency to feeding was observed in both genotypes on the NSF (Fig. 21D). No significant differences were found in the home-cage food consumption during the post-NSF test (WT: 0.14 ± 0.02 g *vs* WT-CORT: 0.11 ± 0.02 g, and KO: 0.16 ± 0.02 g *vs* KO-CORT: 0.11 ± 0.01 g) (Fig. 21E). Therefore, we can discard the influence of changes in locomotion and/or the appetite/motivational drive in that enhancement of anxiety that we observe after chronic corticosterone administration.

Regarding the depressive-related behaviour following chronic corticosterone, both genotypes of mice exhibited an anhedonic response as evidenced by a significant reduction in the sucrose intake in comparison with the respective non-CORT counterparts. KO-CORT mice showed lower values of sucrose intake than WT-CORT mice (WT-CORT: 1.50 ± 0.04 ml *vs* KO-CORT: 1.31 ± 0.07 ml, $p < 0.01$) (Fig. 21F), though the sucrose intake was reduced in a similar magnitude on both corticosterone-treated genotypes (-25% in WT-CORT and -23% in KO-CORT, relative to respective non-CORT counterparts). This depressive-like readout in the

sucrose test was confirmed in the splash test, a paradigm that would resemble the "apathy/self-neglect" symptom of depression (Fig. 21G). Chronic treatment with corticosterone induced a reduction in time of grooming in both genotypes (WT-CORT: -60.1%, *vs* WT $p < 0.05$, and KO-CORT: -52.7%, *vs* KO, $p < 0.05$) (Fig. 21G).

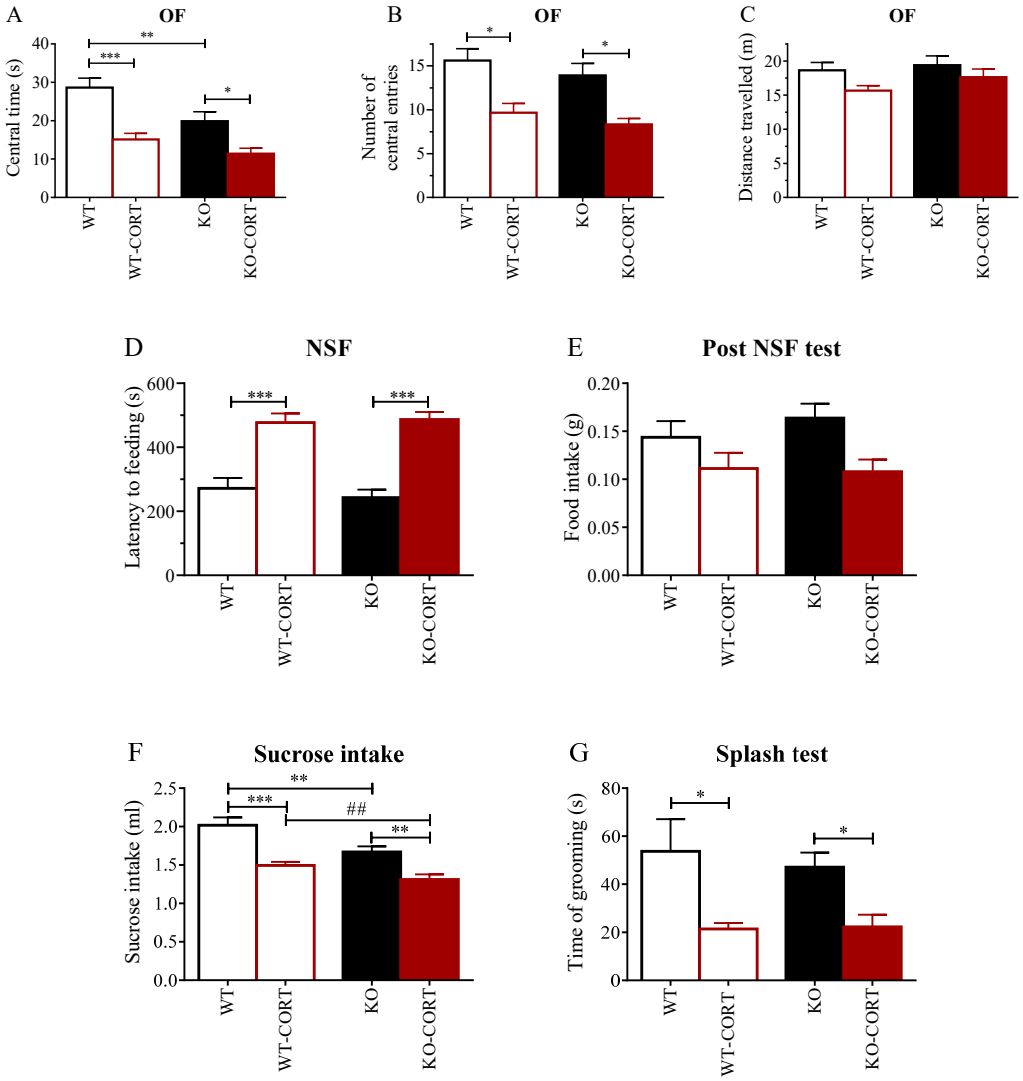


Figure 21. Behavioural effects of chronic corticosterone treatment. Effects of 4 weeks of treatment with corticosterone in central time (**A**), number of central entries (**B**) total distance in the open field (**C**), latency to feeding (**D**) food intake after NSF (**E**), sucrose intake test (**F**) and splash test (**G**) in WT and KO mice. Two-way ANOVA analyses revealed a significant main effect of the model on central time in the OF ($F_{(1,76)}$: 30.3, $p < 0.001$), number of central entries in the OF ($F_{(1,76)}$: 24.2, $p < 0.001$), total distance in the OF ($F_{(1,76)}$: 4.6, $p < 0.05$), latency to feeding ($F_{(1,76)}$: 63.8, $p < 0.001$), food intake after the NSF ($F_{(1,76)}$: 7.9, $p < 0.01$), sucrose intake ($F_{(1,80)}$: 35.2, $p < 0.001$), time of grooming ($F_{(1,80)}$: 13.9, $p < 0.001$) Additionally, the two-way ANOVA analyses also revealed a significant main effect of the genotype in the central time ($F_{(1,76)}$: 9.8, $p < 0.01$) and the sucrose intake ($F_{(1,80)}$: 12.9, $p < 0.001$). Data are mean ± SEM of n = 19-21 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Newman-Keuls *post hoc* test) and $^{\#\#}p < 0.01$ (Student´s *t*-test, unpaired data).

2.1.2. Effect of RS67,333 in the corticosterone model *vs* non-CORT mice

Once confirmed the development of the corticosterone model in WT and 5-HT$_4$ KO mice, the behavioural effects induced by the chronic administration of the 5-HT$_4$ partial agonist RS67,333 (1.5 mg/kg/day, i.p., once daily) were evaluated.

Chronic treatment with RS67,333 was able to revert the enhanced anxiety observed in the open field (induced by chronic corticosterone administration) in WT-CORT mice, but not in KO-CORT mice. In fact, 7-day treatment with RS67,333 induced a significant increase in the number of OF central entries in WT-CORT mice (WT-CORT-RS: +99.1% *vs* WT-CORT values, $p < 0.01$) but not in KO-CORT mice; moreover, this effect of RS67,333 was sustained 7 days later (WT-CORT-RS: +87.1% *vs* WT-CORT at 14-day time-point, $p < 0.01$) (Fig. 22B). In addition, an increased central time was also observed at 7-day time point of treatment (+119.6%) followed by a clear-cut and significant effect of RS67,333 in WT-CORT after 14 days of treatment (WT-CORT-RS: +143.1% *vs* WT-CORT, $p < 0.05$), reaching values similar to those observed in the WT-VH group (Fig. 22A). Thus, confirming the reversion of the "anxiogenic phenotype" induced by chronic corticosterone administration. This anxiolytic effect of RS67,333 in WT-CORT was not due to a sedative-like effect since no changes in locomotor activity (i.e. total distance travelled) were detected (Fig. 22C). A trend to an anxiolytic effect of RS67,333 was also found in non-CORT mice after 14 days of treatment as reflected by the increased number of central entries (WT-RS-14d: +24.4% *vs* WT-VH). However, a transitory anxiogenic effect (reduced central time) was observed after 7 days of treatment in these non-CORT mice (WT-RS-7d: -37.5% *vs* WT-VH, $p < 0.05$, unpaired *t*-test) (Fig. 22A). In KO mice (non-CORT and CORT treated), chronic RS67,333 administration did not change any of the OF readouts (Figs. 22A, B and C).

In a similar way, the effect of the chronic administration of RS67,333 was assessed in the novelty suppressed feeding. Chronic RS67,333 administration did not modify the enhanced anxiety-like response exhibited by both WT- and KO-CORT mice, at any time-point of the treatment (Fig. 23A). Moreover, RS67,333 did not alter the home-cage consumption during the post-NSF test (Fig. 23B). This lack of

behavioural effects of RS67,333 in the NSF was also detected in non-CORT counterparts (Figs. 23A and B).

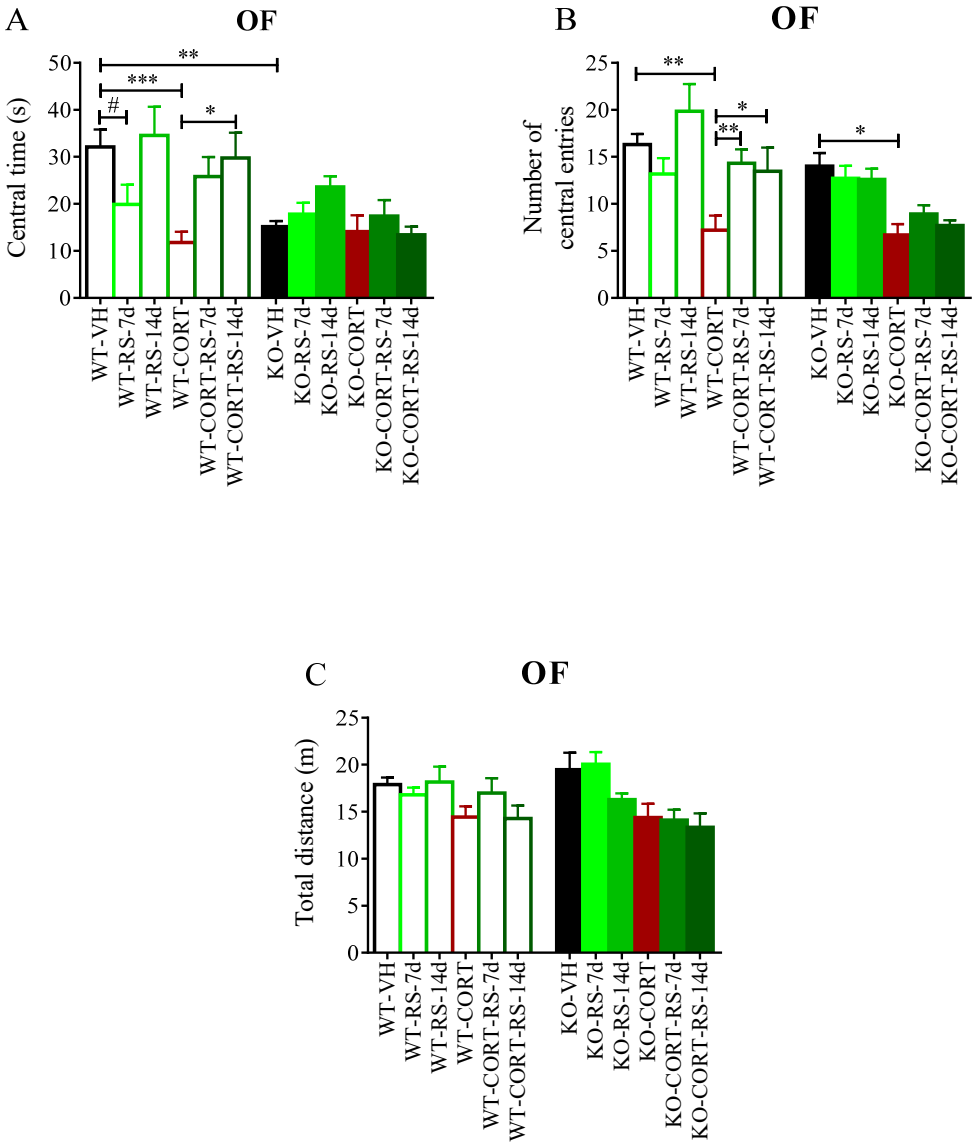


Figure 22. Effects of chronic administration of RS67,333 in the corticosterone model in the open field. Central time (**A**), number of central entries (**B**) and total distance travelled (**C**) were measured after 7 and 14 days (d) of treatment with RS67,333 (RS). Three-way ANOVA analyses revealed a significant interaction effect of model x genotype x treatment on the central time ($F_{(1,116)}$: 3.4, $p < 0.05$). Additionally, two-way interaction effect of model x treatment was found on the number of central entries in the OF ($F_{(2,116)}$: 3.9, $p < 0.05$). Finally, three-way ANOVA analyses also revealed a main effect of the model on the central time ($F_{(1,116)}$: 5.1, $p < 0.05$) and number of central entries ($F_{(1,116)}$: 26.9, $p < 0.001$), a main effect of the genotype on the central time ($F_{(1,116)}$: 14.9, $p < 0.01$) and number of central entries ($F_{(1,116)}$: 13.8, $p < 0.001$) and a main effect of the treatment on the central time ($F_{(2,116)}$: 3.9, $p < 0.05$) in the OF. Data are mean ± SEM of n = 10-11 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Newman-Keuls *post hoc* test) and $^{\#}p < 0.05$ (Student´s *t*-test, unpaired data).

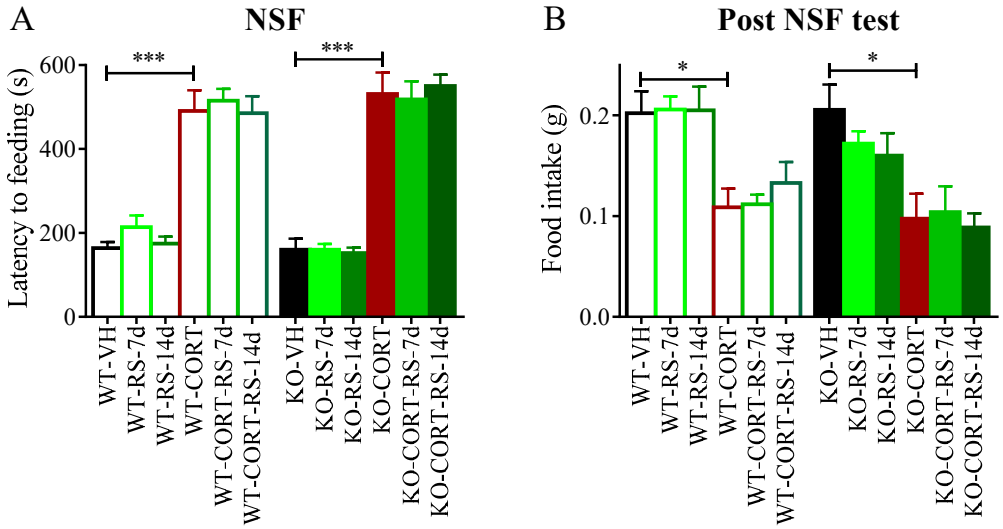


Figure 23. Effects of chronic administration of RS67,333 in the corticosterone model in the novelty suppressed feeding test. Latency to feeding (**A**), and food intake after the NSF (**B**) were measured after 7 and 14 days (d) treatment with RS67,333 (RS) in the NSF test. Three-way ANOVA analyses revealed a significant main effect of the model on the latency to feeding ($F_{(1,104)}$: 383.4, $p < 0.001$) and on the food intake after the test ($F_{(1,104)}$: 54.5, $p < 0.001$) and a significant main effect of the genotype on the food intake after the test ($F_{(1,104)}$: 4.1, $p < 0.05$). Data are mean ± SEM of $n = 9$-10 mice per group. *$p < 0.05$, and ***$p < 0.001$ (Newman-Keuls *post hoc* test).

The aforementioned results demonstrate that the chronic administration of RS67,333 induced a behavioural effect in the OF but not in the NSF test. The former paradigm is used to evaluate the potential anxiolytic activity of drugs whereas the latter one also evaluate potential antidepressant drugs due to its reliable predictive validity. Therefore, and in order to confirm or discard any antidepressant action of RS67,333, we carried out some additional experiments in an extra set of animals. The sucrose intake (anhedonia) and the splash ("self-neglect" or "apathy") tests were used to assess depression-related behaviours. A lower sucrose consumption was detected in both WT (-24.7%) and KO (-15.0%) mice after chronic administration of corticosterone (Fig. 24A), thus, confirming the results described in figure 21F. Additionally, a reduced time of grooming was also found in both genotypes (WT-CORT: -58.7% *vs* WT-VH, $p < 0.05$; KO-CORT: -52.1% *vs* KO-VH, $p < 0.05$) (Fig. 24B). Chronic RS67,333 increased sucrose consumption in KO-CORT but not WT-CORT mice, reflecting an anti-anhedonic effect of the compound in mice lacking 5-HT_4 receptors (KO-CORT-RS: +40.6% *vs* KO-CORT after 7 days of treatment, $p < 0.01$) (Fig. 24A). RS67,333 induced an antidepressant-like action on the splash test as evidenced by an increased grooming time after 7

days of treatment (WT-CORT-RS: +122.4 % *vs* WT-CORT, $p < 0.05$; KO-CORT-RS: +133.9% *vs* KO-CORT, $p < 0.05$) (Fig. 24B). Regarding the effects of RS67,333 treatment in non-CORT mice (WT- and KO-RS), the 5-HT$_4$ receptor partial agonist did not modify the sucrose intake neither the grooming time in comparison with the values of respective counterparts (WT- and KO-VH) (Figs. 24A and B).

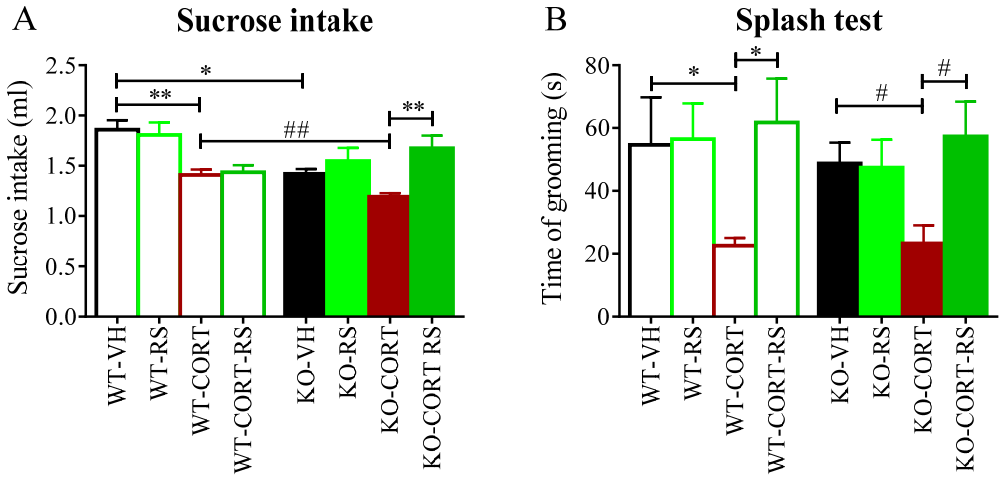


Figure 24. Effects of chronic administration of RS67,333 in the corticosterone model in two depression-related tests. Sucrose intake test (**A**) and splash test (**B**). Three-way ANOVA analyses revealed a genotype x treatment interaction on the sucrose intake ($F_{(1,96)}$: 5.2, $p < 0.05$) and also a model x genotype interaction on the sucrose intake ($F_{(1,96)}$: 6.8, $p < 0.05$). In addition, a significant main effect of treatment ($F_{(1,96)}$: 4.4, $p < 0.05$) and the genotype on the sucrose intake ($F_{(1,96)}$: 6.02, $p < 0.05$) were found. In the splash test, three-way ANOVA revealed a model x treatment interaction on the time of grooming ($F_{(1,65)}$: 6.4, $p < 0.05$) and a main effect of the treatment ($F_{(1,65)}$: 6.6, $p < 0.05$). Data are mean ± SEM of n = 9-13 mice per group. $^{*}p < 0.05$, and $^{**}p < 0.01$ (Newman-Keuls *post hoc* test) and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ (Student´s *t*-test, unpaired data).

2.1.3. Effect of fluoxetine in the corticosterone model *vs* non-CORT mice

The effect of chronic treatment with fluoxetine (25 mg/kg/day, given orally in the drinking water) was assessed in the open field and novelty suppressed feeding tests, and an opposite pattern of responses was encountered. In the open field, chronic fluoxetine was unable to modify significantly the corticosterone-induced anxiety in WT-CORT mice at any time point, although a trend to induce an anxiogenic effect was observed (Figs. 25A and B). This time-dependent anxiogenic effect of fluoxetine was clearly evident in KO-CORT mice as evidenced by the reduced central activity at the 14 day time-point (central time in KO-CORT-flx: -83.1% *vs*

KO-CORT, $p < 0.001$, Fig. 25A; central entries in KO-CORT-flx: -82.3% *vs* KO-CORT, $p < 0.001$; Student´s *t*-test, unpaired data for both). This behavioural outcome of fluoxetine in KO-CORT mice was associated with a decreased locomotor activity (-32.1% in total distance value; KO-CORT-flx-14d: 11.7 m *vs* WT-CORT-flx-14d: 19.5 m, $p < 0.05$) (Fig. 25C). An clear-cut anxiogenic effect of fluoxetine was also found in non-CORT (WT and KO) mice treated with the antidepressant for 14 days (for central time: -53.2% *vs* WT-VH and central entries: -50.2% *vs* WT-VH values, $p < 0.001$ for both parameters) (Figs. 25A and B).

In the novelty suppressed feeding paradigm**,** chronic treatment with fluoxetine completely reverted the characteristic corticosterone-induced enhancement of the anxiety levels, as reflected by a time-dependent pattern of reduction in the latency to feeding in both genotypes. This anxiolytic/antidepressant effect of chronic fluoxetine was already evident after one week of treatment (WT-CORT-flx-7d: -48.7% and KO-CORT-flx-7d: -60.3% *vs* respective CORT-mice, $p <0.001$ for both comparisons) (Fig. 26A). The sustained behavioural effect of fluoxetine was evidenced following 14 days of treatment (WT-CORT-flx-14d: -65.4% and KO-CORT-flx-14d: -75.8% *vs* respective CORT-mice, $p <0.001$ for both comparisons) (Fig. 26A). Chronic fluoxetine also reduced NSF latency of feeding in non-CORT mice (WT-flx-14d: -50.1%, and KO-flx-14d: -48.8%, *vs* respective vehicle groups, $p < 0.05$ for both comparisons) (Fig. 26A). The food intake readouts after the NSF test were not altered, thus, discarding the influence of any motivational/appetite-related factors on the NSF outcome (Fig. 26B).

Regarding the effect of chronic fluoxetine in depression-related tests (i.e sucrose and splash test), it was not possible to carry out these studies due to a shortage of KO and WT subjects. The few available mice were used for the study of the effects of RS67,333 on the corticosterone model above described. Nevertheless, previous studies provide valuable information for comparison purposes. Our group (Vidal *et al.*, 2013) and others (Mendez-David *et al.,* 2014) have reported that the chronic administration of fluoxetine reverses the reduced sucrose consumption and time of grooming exhibited by C57BL/6 mice when subjected to the corticosterone model, findings that demonstrates an anti-anhendonic effect of the antidepressant in this paradigm.

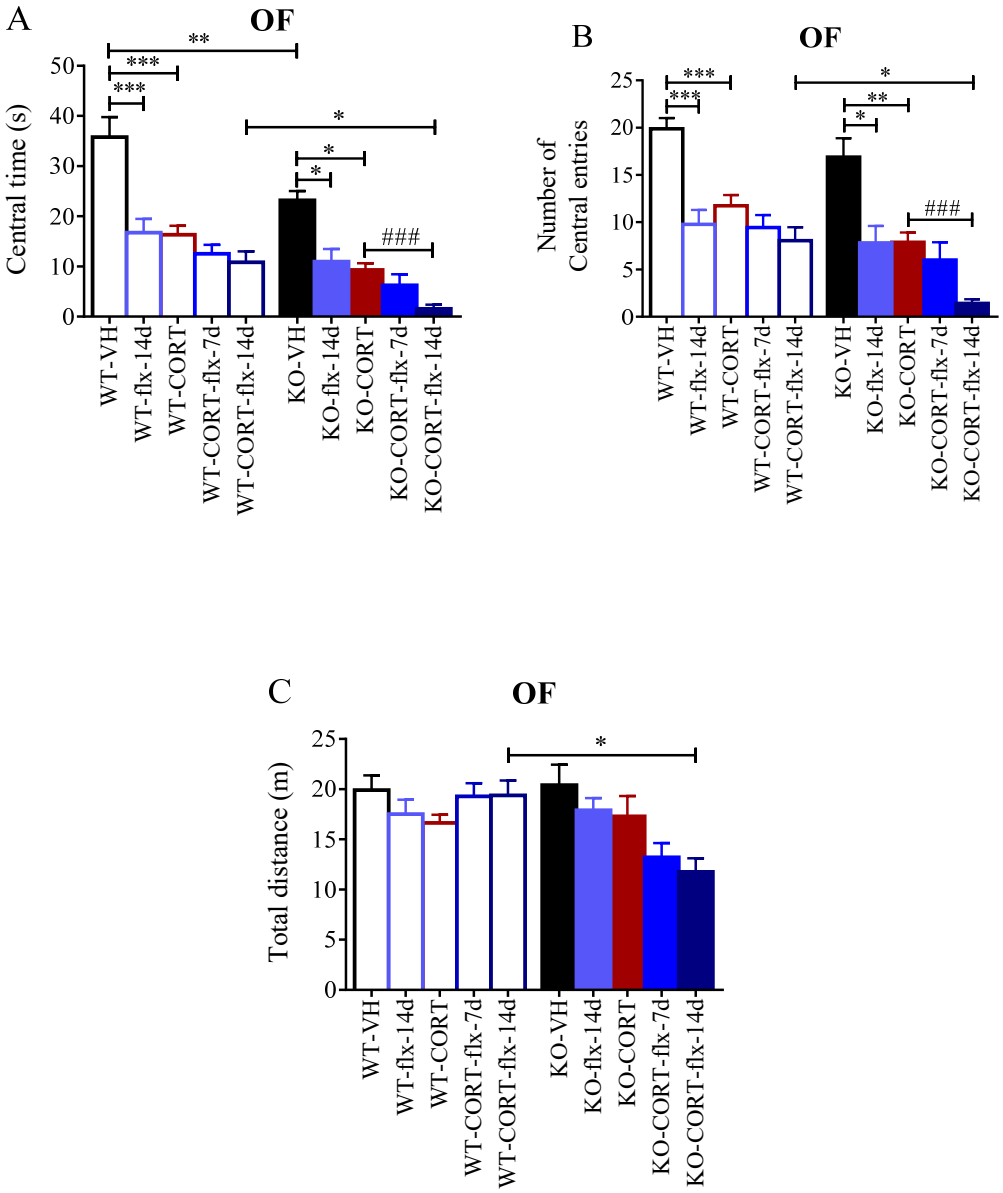


Figure 25. Effects of chronic administration of fluoxetine in the corticosterone model in the open field test. Central time (**A**), number of central entries (**B**) and total distance travelled (**C**) were measured after 7 and 14 days (d) of treatment with fluoxetine (flx) in the open field test. Three-way ANOVA analyses revealed a significant interaction effect of model x treatment on the central time ($F_{(1,117)}$: 6.3, $p < 0.05$). Additionally, three-way ANOVA analyses revealed a significant main effect of the model on the central time ($F_{(1,117)}$: 45.9, $p < 0.001$), number of central entries ($F_{(1,117)}$: 30.6, $p < 0.001$) and total distance travelled ($F_{(1,117)}$: 6.1, $p < 0.05$), a significant main effect of the genotype on the central time ($F_{(1,117)}$: 24.3, $p < 0.001$) the number of central entries ($F_{(1,117)}$: 11.4, $p < 0.01$) and the total distance travelled ($F_{(1,117)}$: 5.6, $p < 0.05$). Moreover, three-way ANOVA analyses also showed a significant main effect of the treatment on the central time ($F_{(2,117)}$: 19.3, $p < 0.001$) and the number of central entries ($F_{(2,117)}$: 20.6, $p < 0.001$). Data are mean ± SEM of n = 12-13 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Newman-Keuls *post hoc* test) and $^{\#\#\#}p < 0.001$ (Student´s *t*-test, unpaired data).

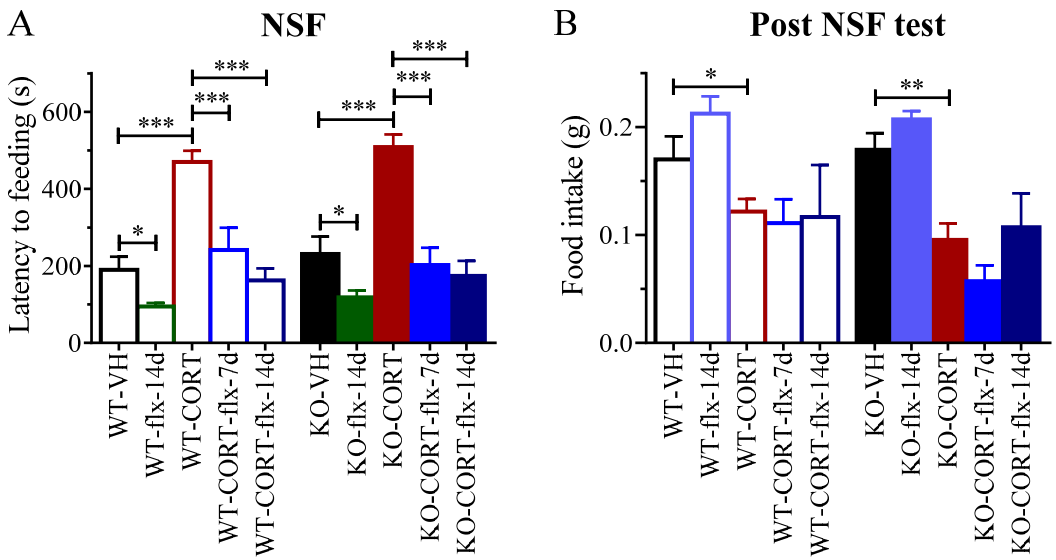


Figure 26. Effects of chronic administration of fluoxetine in the corticosterone model in the novelty suppressed feeding. Latency to feeding (**A**), and food intake after the NSF (**B**) were measured after 7 and 14 days (d) of treatment with fluoxetine (flx) in the NSF test. Three-way ANOVA analyses revealed a significant interaction effect between model and treatment on the latency to feeding ($F_{(1,103)}$: 16.5, $p < 0.001$), a significant main effect of the model on latency to feeding ($F_{(1,103)}$: 40.6, $p < 0.001$) and food intake after the test ($F_{(1,103)}$: 27.8, $p < 0.001$) and a significant main effect of the treatment on the latency to feeding ($F_{(2,103)}$: 39.7, $p < 0.001$). Data are mean ± SEM of n = 11-12 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Newman-Keuls *post hoc* test).

2.2. Neurochemical studies: 5-HT$_{1A}$ receptor functionality

Using [^{35}S]GTPγS binding autoradiography in brain sections, we evaluated whether the corticosterone model and/or the chronic treatment with RS67,333 or fluoxetine could regulate 5-HT$_{1A}$ receptors functionality.

2.2.1. Chronic corticosterone model

In the corticosterone model, we further evaluated the functionality of 5-HT$_{1A}$ receptors across the brain. In midbrain nuclei, a desensitization of the 5-HT$_{1A}$ receptors was found in the DRN in WT-CORT (≈ 44% reduction, tables 5, 6 and Fig. 27B) but not in KO-CORT mice, without detecting changes in the MRN. Regarding the cortical areas, an increase in (±)-8-OH-DPAT stimulated [^{35}S]GPTγS binding was observed in the frontal cortex of KO-CORT mice (≈ 63% increase, tables 5 and 6). The sensitivity of 5-HT$_{1A}$ receptors in hippocampus remained unchanged after chronic corticosterone. Chronic corticosterone administration did

not modify the basal [^{35}S]GTPγS binding across the brain in WT mice, whereas a reduction was detected in some cortical areas in KO mice (data not shown).

2.2.2. Effect of RS67,333 *versus* fluoxetine in the corticosterone model

A brain region-, genotype- and drug-dependent regulation of 5-HT$_{1A}$ receptors functionality in corticosterone-treated mice was observed after the chronic drug administration (for 14 days) of RS67,333 or fluoxetine.

In the brainstem, the desensitization of DRN 5-HT$_{1A}$ autoreceptors observed in corticosterone-treated WT mice was reverted by RS67,333 (WT-CORT-RS: 42.5% *vs* WT-CORT: 17.7% of stimulated [^{35}S]GPTγS binding; $p < 0.05$) (Table 5 and Fig. 27C) achieving values similar to those of WT-VH group (37.8%). By contrast, 5-HT$_{1A}$ autoreceptors desensitization was potentiated by chronic fluoxetine (WT-CORT-flx: 11.5% *vs* WT-CORT: 28.5% of stimulated [^{35}S]GPTγS binding, $p < 0.01$) (Table 6 and Fig. 27D). In addition, fluoxetine fully desensitized MRN 5-HT$_{1A}$ autoreceptors in corticosterone-treated WT mice (WT-CORT-flx: -2% *vs* WT-CORT: 20.9% of stimulated [^{35}S]GPTγS binding, $p < 0.05$) (Table 6 and Fig. 27D). No changes were observed in KO-CORT mice after RS67,333 and fluoxetine treatments, confirming the 5-HT$_4$ receptor dependency of these neurochemical outcomes.

In cortical areas, chronic RS67,333 did not modify the efficacy of coupling of G-proteins to 5-HT$_{1A}$ receptors in any area (Table 5), whereas fluoxetine decreased (±)-8-OH-DPAT stimulated [^{35}S] GTPγS binding in the frontal cortex in both genotypes (WT-CORT-flx: 39.4% *vs* WT-CORT: 88.4%, $p < 0.05$; KO-CORT-flx: 31.7% *vs* KO-CORT: 105.4%, $p < 0.01$) (Table 6).

In the hippocampus, chronic fluoxetine decreased the stimulation of the [^{35}S]GTPγS binding induced by (±)-8-OH-DPAT in the CA1 layers in corticosterone-treated WT mice (WT-CORT-flx: 78.8% *vs* WT-CORT: 118.0%, $p < 0.05$ in CA1oriens; WT-CORT-flx: 98.9% *vs* WT-CORT: 147.3 %, $p < 0.05$ in CA1radiatum) while no changes were observed in KO counterparts (Table 6). Chronic fluoxetine, but not RS67,333, also increased two-fold the (±)-8-OH-DPAT-stimulated [^{35}S]GTPγS

binding in the DG in KO mice (KO-CORT-flx: 74.9% *vs* KO-CORT: 36.5%, $p < 0.05$, Tables 6), whereas no changes were detected in WT-CORT counterparts.

2.2.3. Effect of RS67,333 *versus* fluoxetine in non-CORT mice

In non-CORT mice, chronic administration of RS 67,333 and fluoxetine led to differential changes on 5-HT_{1A} receptors functionality depending on the brain region and genotype.

In the brainstem, RS67,333 desensitized DRN 5-HT_{1A} receptors in WT mice (WT-RS: 17.8% *vs* WT-VH: 37.8%, $p < 0.05$) whereas it increased their functionality in KO counterparts (KO-RS: 29.3% *vs* KO-VH: 14.7%, $p < 0.05$) (Table 5). By contrast, the treatment with fluoxetine did not modify the functionality of DRN 5-HT_{1A} receptors in both genotypes (Table 6).

In cortical areas, chronic RS67,333 reduced the 5-HT_{1A} receptor functionality in the entorhinal cortex in WT (WT-RS: 17.5% *vs* WT-VH: 69.7%, $p < 0.05$) (Table 5) but not in KO mice. Conversely, chronic fluoxetine increased (±)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding in this area on both WT (WT-RS: 104.5% *vs* WT-VH: 72.5%, $p < 0.05$) (Table 6) and KO (KO-RS: 59.3% *vs* KO-VH: 29.7%, $p < 0.05$) (Table 6) mice. Chronic fluoxetine also reduced the 5-HT_{1A} receptor functionality in the frontal cortex WT mice (WT-flx: 33.3% *vs* WT-VH: 85.4%, $p < 0.05$) (Table 6). In the rest of the cortical areas studied, neither RS67,333 (Table 5) nor fluoxetine (Table 6) modified the functionality of the 5-HT_{1A} receptor in non-CORT mice.

In the hippocampus, chronic RS67,333 treatment was not associated with significant changes in 5-HT_{1A} receptor functionality in the hippocampus in non-CORT animals of both genotypes (Table 5). However, chronic fluoxetine, in both genotypes, reduced the level of (±)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding across the hippocampus, although a higher desensitization was observed in KO mice, especially in the DG (Table 6).

Table 5. Effect of chronic RS67,333 treatment on the % stimulation of specific [^{35}S]GTPγS binding induced by (±)-8-OH-DPAT in the corticosterone model and non-CORT mice. Three-way ANOVA analyses revealed an interaction effect of model x genotype x treatment in DRN ($F_{(1,46)}$: 18.3, $p < 0.001$) and entorhinal cortex ($F_{(1,40)}$: 6.9, $p < 0.01$). It also showed an interaction effect of model x treatment in DRN ($F_{(1,46)}$: 4.8, $p < 0.05$) and an interaction effect of genotype x treatment in entorhinal cortex ($F_{(1,40)}$: 4.8, $p < 0.05$). A main effect of genotype in entorhinal cortex ($F_{(1,40)}$: 6.3, $p < 0.05$), and FrCx ($F_{(1,46)}$: 5.8, $p < 0.05$) were found. DRN: dorsal raphe nucleus, MRN: median raphe nucleus, mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Ent Cx: entorhinal cortex, CA1 oriens: CA1 oriens hippocampal field, CA1 rad: CA1 radiatum hippocampal field, CA3: CA3 hippocampal field, and DG: dentate gyrus. Data are mean ± SEM of n = 6-8 animals per group and values are expressed as percentage of (±)-8-OH-DPAT stimulated [^{35}S]GTPγS binding. *$p < 0.05$ *vs* WT-VH; $^{+}p < 0.05$ *vs* WT-CORT; $^{\&}p < 0.05$ *vs* KO-VH (Newman-Keuls *post hoc* test).

	WT-VH	WT-RS	WT-CORT	WT-CORT-RS	KO-VH	KO-RS	KO-CORT	KO-CORT-RS
Brainstem areas								
DRN	37.8 ± 5.1	**17.8 ± 7.5***	**17.7 ± 3.8***	**42.5 ± 6.3^{+}**	**14.7 ± 3.7***	**29.3 ± 4.7$^{\&}$**	21.6 ± 6.4	22.2 ± 7.2
MRN	18.4 ± 4.8	11.9 ± 9.8	20.2 ± 7.8	11.8 ± 6.3	14.8 ± 4.9	7.5 ± 3.1	7.3 ± 7.1	9.0 ± 4.8
Cortical areas								
mPFrCx	63.1 ± 9.3	44.2 ± 11.7	40.3 ± 8.4	57.0 ± 17.4	39.3 ± 11.3	44.8 ± 10.4	67.2 ± 12.4	55.9 ± 10.8
FrCx	87.4 ± 9.3	90.4 ± 16.9	93.7 ± 11.9	130.4 ± 35.5	74.3 ± 12.4	94.5 ± 14.3	**123.5 ± 12.6$^{\&}$**	107.0 ± 16.3
Ent Cx	69.7 ± 11.5	**17.5 ± 2.6***	54.2 ± 10.0	53.3 ± 26.8	**22.5 ± 6.4***	37.8 ± 7.4	35.4 ± 15.2	28.5 ± 8.3
Hippocampal areas								
CA1 oriens	129.2 ± 14.6	99.7 ± 16.5	108.3 ± 10.6	135.6 ± 21.7	140.5 ± 11.5	146.3 ± 20.3	128.8 ± 9.3	107.2 ± 14.3
CA1 rad	132.0 ± 12.7	121.8 ± 15.0	115.2 ± 7.6	125.6 ± 16.5	136.5 ± 11.0	137.6 ± 13.3	139.2 ± 10.4	109.1 ± 9.6
CA3	33.0 ± 9.6	42.9 ± 12.9	37.5 ± 3.5	56.2 ± 14.9	36.2 ± 5.8	31.5 ± 6.8	40.9 ± 11.3	32.9 ± 7.4
DG	52.2 ± 8.5	57.5 ± 9.0	45.5 ± 5.1	71.2 ± 18.5	55.2 ± 8.9	48.5 ± 4.6	51.7 ± 10.3	35.9 ± 9.3

Table 6. Effect of chronic fluoxetine treatment on the % stimulation of specific [^{35}S]GTPγS binding induced by (±)-8-OH-DPAT in the corticosterone model and non-CORT mice. Three-way ANOVA analyses revealed an interaction of model x genotype x treatment in MRN ($F_{(1,46)}$: 5.7, $p < 0.05$), CA1 oriens ($F_{(1,49)}$: 5.8, $p < 0.05$) and DG ($F_{(1,43)}$: 6.1, $p < 0.05$). Additionally, it showed a two way interaction of model x genotype in DRN ($F_{(1,50)}$: 15.4, $p < 0.001$) and entorhinal cortex ($F_{(1,51)}$: 7.8, $p < 0.01$). An interaction effect of model x treatment in CA1 oriens ($F_{(1,49)}$: 5.3, $p < 0.05$), CA1 radiatum ($F_{(1,43)}$: 4.3, $p < 0.05$) and dentate gyrus ($F_{(1,43)}$: 22.7, $p < 0.001$) was found. A main effect of model in DRN ($F_{(1,50)}$: 15.4, $p < 0.001$), entorhinal cortex ($F_{(1,51)}$: 4.7, $p < 0.05$), and DG ($F_{(1,43)}$: 27.3, $p < 0.001$) was found. A significant main effect of treatment in MRN ($F_{(1,46)}$: 4.1, $p < 0.05$), FrCx ($F_{(1,56)}$: 37.9, $p < 0.001$), CA1 oriens ($F_{(1,49)}$: 19.8, $p < 0.001$) and CA1 rad ($F_{(1,43)}$: 38.3, $p < 0.001$) was found. Finally, a main effect of genotype was found in the CA1 oriens ($F_{(1,49)}$: 4.4, $p < 0.05$). DRN: dorsal raphe nucleus, MRN: median raphe nucleus, mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Ent Cx: entorhinal cortex, CA1 oriens: CA1 oriens hippocampal field, CA1 rad: CA1 radiatum hippocampal field, CA3: CA3 hippocampal field, and DG dentate gyrus. Data are mean ± SEM of n = 6-8 animals per group and values are expressed as percentage of (±)-8-OH-DPAT stimulated [^{35}S]GTPγS binding. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* WT-VH; $^{+}p < 0.05$ and $^{++}p < 0.01$ *vs* WT-CORT; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ and $^{\&\&\&}p < 0.001$*vs* KO-VH; $^{@}p < 0.05$ *vs* WT-flx; $^{\$}p < 0.05$ *vs* WT-CORT-flx; $^{€}p < 0.05$ and $^{€€}p < 0.01$ *vs* KO-CORT (Newman-Keuls *post hoc* test) and $^{\#\#}p < 0.01$ *vs* WT-VH (Student's *t*-test, unpaired data).

	WT-VH	WT- flx	WT-CORT	WT-CORT-flx	KO-VH	KO-flx	KO-CORT	KO-CORT-flx
Brainstem areas								
DRN	44.3 ± 3.1	33.5 ± 8.7	**28.5 ± 4.3***	**11.5 ± 4.5^{++}**	**26.9 ± 4.8$^{\#\#}$**	37.5 ± 11.2	30.3 ± 3.5	25.9 ± 6.7
MRN	23.3 ± 5.0	17.9 ± 6.0	20.9 ± 5.3	**-2.0 ± 7.2^{+}**	29.6 ± 4.8	13.3 ± 6.4	12.0 ± 5.9	**23.8 ± 6.7$^{\$}$**
Cortical areas								
mPFrCx	64.3 ± 21.0	35.9 ± 18.6	54.1 ± 16.7	37.0 ± 16.9	38.5 ± 5.2	20.5 ± 27.7	52.4 ± 16.1	21.3 ± 13.7
FrCx	85.4 ± 8.3	**33.3 ± 9.2***	88.4 ± 14.7	**39.4 ± 8.4^{+}**	65.8 ± 9.4	39.8 ± 10.6	**105.4 ± 13.8$^{\&}$**	**31.7 ± 10.7$^{€€}$**
Ent Cx	72.5 ± 6.6	**104.5 ± 10.3***	44.5 ± 10.2	36.9 ± 18.8	**29.7 ± 6.9****	**59.3 ± 20.0$^{\&@}$**	36.3 ± 10.2	54.3 ± 23.8
Hippocampal areas								
CA1 oriens	123.4 ± 14.7	**77.7 ± 10.6***	118.0 ± 15.7	**78.8 ± 9.7^{+}**	110.9 ± 10.3	**34.5 ± 4.6$^{\&\&@}$**	88.2 ± 19.1	97.0 ± 15.6
CA1 rad	129.1 ± 12.0	**79.7 ± 16.2***	143.7 ± 9.9	**98.9 ± 7.2^{+}**	123.6 ± 9.2	**46.6 ± 11.5$^{\&\&}$**	118.1 ± 11.7	101.4 ± 12.0
CA3	29.6 ± 9.4	17.1 ± 4.3	18.9 ± 5.4	19.8 ± 8.2	14.1 ± 7.0	18.4 ± 11.7	35.8 ± 23.3	32.6 ± 10.6
DG	50.7 ± 7.0	**17.9 ± 6.0*****	59.6 ± 10.7	51.5 ± 8.7	40.4 ± 2.2	**1.1 ± 10.0$^{\&\&\&}$**	36.5 ± 8.3	**74.9 ± 10.8$^{€}$**

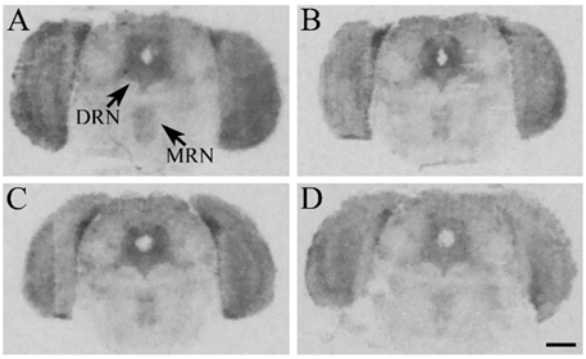


Figure. 27. **Representative autoradiograms of (±)-8-OH-DPAT stimulated [^{35}S]GTPγS binding in the corticosterone model at the level of midbrain**. WT-VEH (**A**), WT-CORT (**B**), WT-CORT-RS (**C**) and WT-CORT-flx (**D**). DRN: dorsal raphe nucleus, MRN: median raphe nucleus. Scale bar = 1 mm.

2.3. Molecular studies: mRNA expression of neuroplasticity markers

In situ hybridization studies were carried out using coronal brain sections obtained from WT and KO mice in order to evaluate whether the corticosterone model and/or the chronic treatment with RS67,333 or fluoxetine could modulate the levels of mRNA expression of neuroplascity proteins related with depression and antidepressants effects: BDNF/trkB and *Arc*.

2.3.1. BDNF mRNA expression

2.3.1.1. Chronic corticosterone model

In both genotypes of corticosterone-treated mice, levels of BDNF mRNA expression were not different to those found in their respective vehicle-treated groups, in all the brain areas analyzed. However, 5-HT$_4$ KO mice administered corticosterone exhibited a lower BDNF mRNA expression than their WT-counterparts in some cortico-hippocampal areas: mPFrCx (≈ 30% reduction in KO-CORT *vs* WT-CORT, $p < 0.05$), CA1 (≈ 34% reduction in KO-CORT *vs* WT-CORT, $p < 0.01$), and DG (≈ 22% reduction in KO-CORT *vs* WT-CORT, $p < 0.01$) (Tables 7 and 8). In others brain areas, the levels of BDNF mRNA expression were similar between WT and KO mice administered chronic corticosterone.

2.3.1.2. Effect of RS67,333 versus fluoxetine in the corticosterone model

Chronic drugs administration regulated BDNF mRNA expression only in the hippocampus, and in a drug- and genotype-dependent manner.

In cortical areas, neither RS67,333 nor fluoxetine affected the levels of BDNF mRNA expression in WT or KO mice administered corticosterone.

In the hippocampus, chronic RS67,333 reduced slightly, but significantly, BDNF mRNA expression in the CA1 field and the DG in WT-CORT mice but not in KO-CORT mice (CA1 field, WT-CORT-RS: -25.2% *vs* WT-CORT, $p < 0.05$; DG, WT-CORT-RS: -19.6% *vs* WT-CORT, $p < 0.05$) (Table 7 and Fig. 28C). Conversely, chronic administration of fluoxetine increased BDNF mRNA expression in the DG in corticosterone-treated mice of both genotypes (WT-CORT-flx: +40.0% *vs* WT-CORT, $p < 0.001$; KO-CORT-flx: +55.5 % *vs* KO-CORT, $p < 0.01$) (Table 8 and Fig. 28D). In addition, we detected a significant difference on BDNF mRNA expression in the CA1 field between fluoxetine-treated WT-CORT and KO-CORT mice (KO-CORT-flx: -34.3% *vs* WT-CORT-flx, $p < 0.05$) (Table 8).

2.3.1.3. Effect of RS67,333 versus fluoxetine in non-CORT mice

The 5-HT_4 partial agonist RS67,333 exerted a differential effect upon the BDNF mRNA expression in the CA1 hippocampal field depending on the genotype: a downregulation in WT (WT-RS: -22.4% *vs* WT-VH, $p < 0.05$) and an upregulation in KO (KO-RS: +41.4% *vs* KO-VH, $p < 0.01$) mice (Table 7). In the case of fluoxetine, a significant reduction of the BDNF levels was observed in the DG of KO mice (KO-flx: -32.1% *vs* KO-VH, $p < 0.05$) and no changes were observed in WT mice (Table 8).

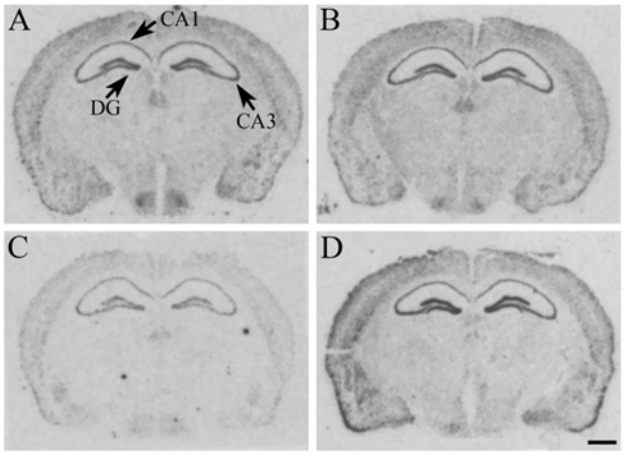


Figure 28. Autoradiograms showing the hippocampal BDNF mRNA expression by *in situ* hybridization in the corticosterone model. WT-VH (**A**), WT-CORT (**B**), WT-CORT-RS (**C**) and WT-CORT-flx (**D**). CA1 and CA3: CA1 and CA3 fields of the hippocampus and DG: dentate gyrus. Scale bar = 1 mm.

Table 7. Effect of chronic RS67,333 treatment in BDNF mRNA levels in the corticosterone model and non-CORT mice. Three way ANOVA analyses revealed an interaction of genotype x treatment in mPFrCx ($F_{(1,38)}$: 10.5, $p < 0.01$), CA1 ($F_{(1,41)}$: 33.5, $p < 0.001$) and DG ($F_{(1,47)}$: 11.1, $p < 0.01$). It also revealed an interaction of model x genotype in mPFCx ($F_{(1,38)}$: 8.8, $p < 0.001$) and CA1 ($F_{(1,41)}$: 6.0, $p < 0.05$), and an interaction of model x treatment in CA1 ($F_{(1,41)}$: 4.5, $p < 0.05$). A significant main effect of model in CA1 ($F_{(1,41)}$: 6.3, $p < 0.01$) and a main effect of genotype in DG ($F_{(1,47)}$: 5.3, $p < 0.01$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, CA1: CA1 hippocampal field, CA3: CA3 hippocampal field, and DG dentate gyrus. Data are mean ± SEM (nCi/g tissue) of n : 5-7 mice per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* WT-VH, $^{+}p < 0.05$ and $^{++}p < 0.01$ *vs* WT-CORT, $^{@}p < 0.05$ *vs* WT-RS (Newman-Keuls post hoc test) and $^{\#}p < 0.05$ *vs* WT-CORT (Student´s *t*-test, unpaired data).

	WT-VH	WT-RS	WT-CORT	WT-CORT-RS	KO-VH	KO-RS	KO-CORT	KO-CORT-RS
Cortical areas								
mPFrCx	15.2 ± 1.4	11.2 ± 2.7	17.5 ± 1.5	15.6 ± 1.6	13.9 ± 1.3	**18.0 ± 0.8**$^{@}$	**12.4 ± 1.3**$^{\#}$	15.7 ± 1.0
FrCx	14.3 ± 1.4	11.5 ± 1.4	16.5 ± 1.1	14.8 ± 1.3	12.1 ± 1.3	13.0 ± 1.4	14.9 ± 2.2	14.0 ± 1.4
Cing Cx	10.5 ± 1.3	9.0 ± 1.0	12.1 ± 1.1	10.1 ± 1.4	9.7 ± 1.2	10.6 ± 1.6	10.9 ± 1.1	13.1 ± 1.3
Pyr Cx	17.7 ± 0.9	16.4 ± 1.7	21.8 ± 1.2	21.3 ± 1.1	18.8 ± 1.6	21.8 ± 1.6	17.9 ± 1.6	19.5 ± 2.8
Hippocampal areas								
CA1	19.4 ± 1.0	**15.1 ± 1.1***	20.5 ± 0.8	**15.3 ± 2.7**$^{+}$	**15.3 ± 1.1****	**21.6 ± 0.8**$^{\&\&\&}$	**13.5 ± 0.9**$^{++}$	15.2 ± 1.5
CA3	30.5 ± 1.6	28.6 ± 1.0	29.8 ± 1.8	25.4 ± 4.2	30.2 ± 2.5	35.4 ± 1.7	26.2 ± 1.2	25.1 ± 2.1
DG	38.8 ± 1.8	35.6 ± 2.4	39.8 ± 0.8	**32.0 ± 3.3**$^{+}$	32.6 ± 1.8	37.2 ± 2.1	**31.0 ± 1.1**$^{++}$	33.1 ± 1.8

Table 8. Effect of chronic fluoxetine treatment in BDNF mRNA levels in the corticosterone model and non-CORT mice. Three-way ANOVA analyses revealed an interaction of model x treatment in DG ($F_{(1,54)}$: 19.5, $p < 0.001$). It also revealed a main effect of genotype in mPFrCx ($F_{(1,45)}$: 9.1, $p < 0.05$), CA1 ($F_{(1,55)}$: 33.4, $p < 0.001$) and DG ($F_{(1,54)}$: 11.3, $p < 0.01$), a main effect of treatment in DG ($F_{(1,54)}$: 11.6, $p < 0.01$), and a main effect of model in DG ($F_{(1,54)}$: 27.9, $p < 0.001$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, CA1: CA1 hippocampal field, CA3: CA3 hippocampal field, and DG dentate gyrus. Data are mean ± SEM (nCi/g tissue) of *n* : 6-8 mice per group. $^{+}p < 0.05$ and $^{+++}p < 0.001$ *vs* WT-CORT, $^{€€}p < 0.01$ *vs* KO-CORT, $^{\&}p < 0.05$ *vs* KO-VH, $^{@}p < 0.05$ *vs* WT-flx, $^{\$}p < 0.05$ *vs* WT-CORT-flx (Newman-Keuls post hoc test) and $^{\#}p < 0.05$ *vs* WT-CORT (Student´s *t*-test, unpaired data).

	WT-VH	WT- flx	WT-CORT	WT-CORT-flx	KO-VH	KO-flx	KO-CORT	KO-CORT-flx
Cortical areas								
mPFrCx	14.4 ± 1.2	18.6 ± 4.1	14.8 ± 1.1	17.0 ± 3.7	11.8 ± 0.7	11.8 ± 1.8	**10.2 ± 1.6**$^{+}$	12.9 ± 1.8
FrCx	14.0 ± 1.0	12.5 ± 1.2	13.3 ± 1.0	18.1 ± 4.5	10.7 ± 1.0	9.1 ± 1.2	10.1 ± 1.0	13.5 ± 2.0
Cing Cx	11.3 ± 0.6	14.2 ± 1.6	11.9 ± 1.2	11.1 ± 2.3	12.2 ± 1.7	11.4 ± 1.1	12.0 ± 1.2	10.8 ± 1.5
Pyr Cx	18.0 ± 2.3	26.2 ± 3.4	19.9 ± 3.3	16.3 ± 2.3	22.8 ± 3.0	21.2 ± 1.2	23.1 ± 3.2	24.2 ± 3.6
Hippocampal areas								
CA1	18.8 ± 1.2	19.9 ± 1.0	20.2 ± 1.3	18.4 ± 2.0	15.8 ± 1.2	14.2 ± 0.8	**14.4 ± 0.9**$^{+}$	**12.1 ± 1.4**$^{\$}$
CA3	31.0 ± 3.5	30.2 ± 2.0	29.1 ± 2.4	33.0 ± 5.3	30.8 ± 4.3	26.6 ± 2.6	27.4 ± 2.6	25.5 ± 4.2
DG	41.4 ± 3.2	48.9 ± 3.6	47.0 ± 2.4	**67.7 ± 5.7**$^{+++}$	40.5 ± 2.6	**27.5 ± 3.6**$^{\&@}$	**39.3 ± 2.5**$^{\#}$	**61.1 ± 8.4**$^{€€}$

A correlation analysis was performed between NSF latency and BDNF mRNA expression in the dentate gyrus (plotting *all groups* indicated in tables 7 and 8). As shown in figure 29A, the NSF latency to feeding correlated negatively with hippocampal BDNF mRNA expression (higher BDNF expression is linked to a lower anxiety, r^2: 0.103, $p < 0.001$). We further analyzed the correlation between the NSF outcome and BDNF mRNA expression in the hippocampal DG in the *corticosterone treated mice* (Figs. 29B and C). A significant correlations were encountered in WT-CORT treated with fluoxetine and its vehicle (r^2: 0.465, $p < 0.001$, Fig. 29C) but not in those mice treated with RS67,333 and its vehicle (r^2: 0.035, ns, Fig 29B).

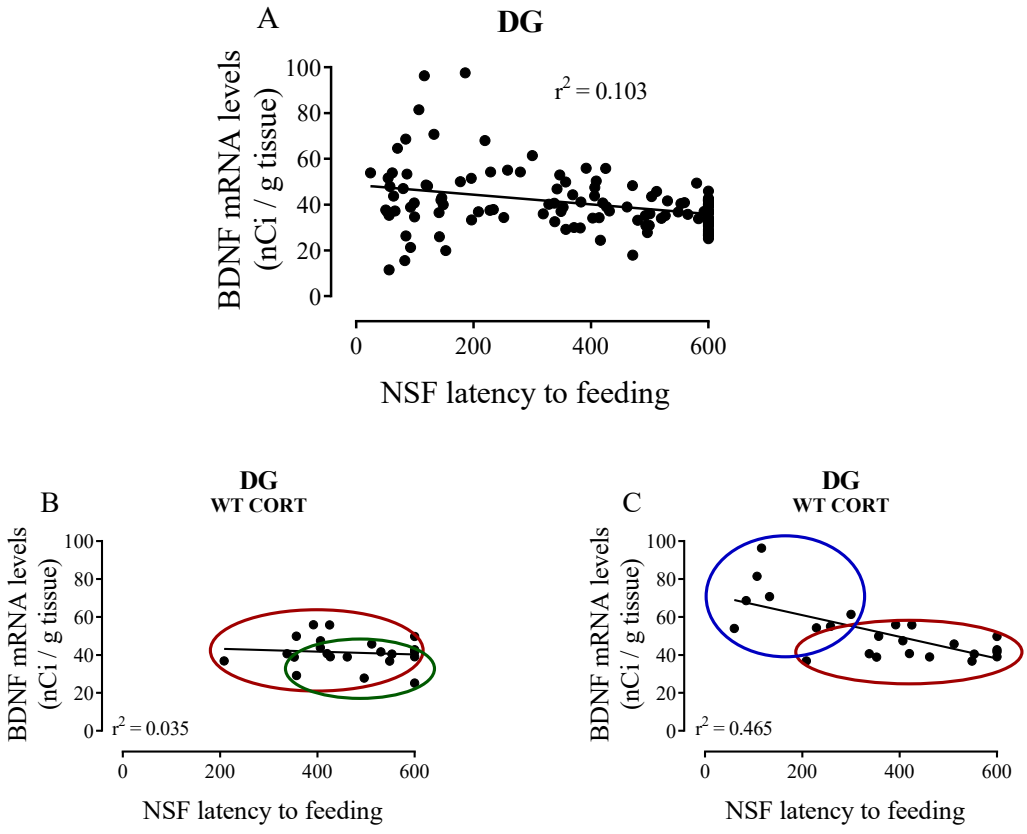


Figure 29. Correlation between the levels of BDNF mRNA expression in the dentate gyrus and the novelty suppressed feeding readouts. The regression lines for BDNF mRNA expression *versus* the latency to feeding in the NSF. Plotting all the experimental groups (**A**), WT-CORT mice treated with RS67,333 (green circle) or its vehicle (red circle) (**B**) and WT-CORT mice treated with fluoxetine (blue circle) or its vehicle (red circle) (**C**). Note the inverse relationship between BDNF mRNA expression and the NSF latency to feeding values (the higher expression of BDNF, the lower anxiety in the NSF) in WT-CORT mice treated with fluoxetine but not WT-CORT mice treated with RS67,333.

2.3.2. TrkB mRNA expression

2.3.2.1. Chronic corticosterone model

After chronic corticosterone administration, levels of trkB mRNA expression in WT and KO-mice were similar to those values observed in their respective non-CORT counterparts across the brain (Tables 9 and 10).

2.3.2.2. Effect of RS67,333 versus fluoxetine in the corticosterone model

When comparing the effect of both drugs in corticosterone-treated mice, only the chronic administration of the 5-HT_4 partial agonist was able to modulate the expression of TrkB mRNA; in fact, RS67,333 did reduce it only in the nucleus accumbens and only in WT-CORT (WT-CORT-RS: -18.8% *vs* WT-CORT, $p < 0.05$) (Table 9) but not in KO-CORT mice. Chronic administration of fluoxetine was not associated with changes on TrkB mRNA expression all throughout the brain (Table 10).

2.3.2.3. Effect of RS67,333 versus fluoxetine in non-CORT mice

Similarly, chronic RS67,333 decreased the levels of trkB mRNA expression only in the nucleus accumbens in WT mice (WT-RS: -40.1% *vs* WT-VH, $p < 0.001$) (Table 7), a molecular readout not detected in KO counterparts. Chronic administration of fluoxetine in non-CORT mice was not associated with changes on TrkB mRNA expression, all throughout the brain (Tables 9 and 10).

Table 9. Effects of chronic RS67,333 treatment in TrkB mRNA levels in the corticosterone model and non-CORT mice. Three-way ANOVA analyses revealed a two way interaction effect of genotype x treatment ($F_{(1,44)}$: 5.7, $p < 0.05$), and a main effect of treatment in nucleus accumbens ($F_{(1,44)}$: 8.3, $p < 0.01$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, Nuc acc: nucleus accumbens, CA1: CA1 hippocampal field, CA3: CA3 hippocampal field, and DG: dentate gyrus. Data are mean ± SEM (nCi/g tissue) of n = 6-9 mice per group. $^{***}p < 0.001$ *vs* WT-VH, $^{+}p < 0.05$ *vs* WT-CORT (Newman-Keuls *post hoc* test).

	WT-VH	WT-RS	WT-CORT	WT-CORT-RS	KO-VH	KO-RS	KO-CORT	KO-CORT-RS
Cortical areas								
mPFrCx	65.6 ± 7.0	59.8 ± 8.2	71.6 ± 4.7	62.3 ± 4.7	79.0 ± 7.8	76.4 ± 7.4	79.1 ± 8.3	92.8 ± 3.8
FrCx	42.6 ± 4.5	40.8 ± 5.4	53.7 ± 2.8	48.9 ± 4.0	52.7 ± 4.8	50.5 ± 6.5	53.6 ± 2.4	58.7 ± 5.0
Cing Cx	86.8 ± 5.9	66.5 ± 6.7	76.1 ± 3.8	62.4 ± 5.4	72.4 ± 6.7	67.8 ± 4.9	69.9 ± 7.2	64.3 ± 6.2
Pyr Cx	108.9 ± 9.5	80.0 ± 11.4	118.1 ± 4.0	101.7 ± 7.0	93.8 ± 11.3	69.7 ± 9.6	92.0 ± 6.3	92.3 ± 12.8
Limbic areas								
Nuc acc	53.2 ± 3.0	**31.9 ± 2.7*****	53.0 ± 2.4	**43.0 ± 2.0**$^{+}$	48.8 ± 5.2	42.7 ± 5.0	48.8 ± 3.1	51.8 ± 7.1
Amygdala	61.3 ± 4.4	62.9 ± 6.6	69.1 ± 6.9	82.3 ± 10.2	70.6 ± 4.4	79.6 ± 6.8	59.5 ± 5.8	63.9 ± 6.3
Hypothalamus	85.4 ± 12.0	76.5 ± 9.4	79.8 ± 13.6	105.0 ± 20.5	106.0 ± 7.0	94.7 ± 10.1	92.8 ± 11.9	78.1 ± 13.8
Hippocampus								
CA1	79.0 ± 8.6	68.2 ± 10.4	69.2 ± 7.6	75.6 ± 5.0	85.8 ± 6.1	82.8 ± 4.3	71.8 ± 4.3	90.2 ± 8.1
CA3	75.8 ± 8.3	65.4 ± 8.2	70.9 ± 8.6	77.8 ± 10.1	87.0 ± 7.3	77.9 ± 5.8	72.6 ± 8.6	82.9 ± 11.7
DG	80.0 ± 11.0	67.9 ± 7.6	76.3 ± 9.3	70.7 ± 8.4	79.0 ± 5.2	90.4 ± 4.6	69.6 ± 7.3	85.6 ± 7.8

Table 10. Effect of chronic fluoxetine treatment in TrkB mRNA levels in the corticosterone model and non-CORT mice. mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, Nuc acc: nucleus accumbens CA1: CA1 hippocampal field, CA3: CA3 hippocampal field, and DG: dentate gyrus. Data are mean ± SEM (nCi/g tissue) of *n* = 6-9 mice per group.

	WT-VH	WT-flx	WT-CORT	WT-CORT-flx	KO-VH	KO-flx	KO-CORT	KO-CORT-flx
Cortical areas								
mPFrCx	92.6 ± 5.5	98.2 ± 6.2	94.1 ± 5.0	105.3 ± 3.9	93.8 ± 5.1	96.8 ± 7.8	105.2 ± 4.9	88.6 ± 5.8
FrCx	68.6 ± 4.4	69.9 ± 5.2	79.3 ± 4.7	90.0 ± 3.7	75.8 ± 6.8	78.8 ± 6.2	75.6 ± 3.2	79.2 ± 6.4
Olf Cx	117.5 ± 8.8	116.3 ± 9.3	126.6 ± 2.5	112.2 ± 4.4	112.2 ± 4.1	112.8 ± 9.2	104.7 ± 4.2	111.6 ± 9
Cing Cx	86.9 ± 4.5	82.2 ± 7.6	98.8 ± 5.2	100.0 ± 4.4	94.5 ± 4.3	95.9 ± 7.2	105.2 ± 4.2	91.9 ± 4.7
Pyr Cx	108.5 ± 4.4	104.2 ± 7.6	122.3 ± 8.5	112.6 ± 7.5	109.1 ± 4.7	104.7 ± 8.4	125.5 ± 9.5	111.4 ± 8.7
Limbic areas								
Nuc acc	57.0 ± 3.6	53.0 ± 4.5	60.4 ± 4.1	67.7 ± 6.3	60.1 ± 3.4	55.7 ± 5.6	64.3 ± 3.2	62.5 ± 4.0
Amygdala	88.6 ± 5.6	81.6 ± 4.9	86.9 ± 6.2	84.3 ± 3.5	83.1 ± 3.7	81.6 ± 4.9	82.8 ± 4.2	72.9 ± 2.7
Hypothalamus	102.1 ± 7.6	106.6 ± 4.8	93.7 ± 8.6	88.9 ± 5.2	94.3 ± 7.0	93.4 ± 5.7	89.7 ± 7.8	80.1 ± 6.0
Hippocampus								
CA1	95.6 ± 4.6	101.6 ± 5.7	96.3 ± 7.2	93.5 ± 4.9	88.7 ± 4.4	90.5 ± 3.9	96.8 ± 5.0	83.7 ± 4.3
CA3	100.4 ± 4.2	103.9 ± 7.8	89.0 ± 6.6	96.9 ± 4.6	94.0 ± 3.5	100.0 ± 4.6	95.4 ± 5.1	88.5 ± 6.6
DG	101.2 ± 4.9	110.6 ± 8.5	101.8 ± 6.9	110.5 ± 5.7	93.6 ± 7.2	101.1 ± 4.7	102.3 ± 4.4	91.7 ± 6.6

2.3.3. *Arc* mRNA expression

2.3.3.1. Chronic corticosterone model

Significant changes were observed in some cortical areas, in KO but not WT mice administered corticosterone. Indeed, a significant decrease in the levels of *Arc* mRNA expression was detected in mPFrCx (~ -46%) in KO-CORT mice.

2.3.3.2. Effect of RS67,333 versus fluoxetine in the corticosterone model

When comparing the effect of both drugs in corticosterone-treated mice, a striking differential drug-dependent modulation of *Arc* mRNA expression was encountered. The molecular effects of RS67,333 were restricted to the CA3 hippocampal field (Table 11), whereas the effects of fluoxetine were detected in several brain areas (Table 12).

In cortical areas, chronic fluoxetine treatment, in WT mice, was associated with a downregulation of *Arc* mRNA expression in the mPFrCx (WT-CORT-flx: -35.1% *vs* WT-CORT, $p < 0.01$) (Table 12). In the cingulate cortex, a downregulation were observed in both genotypes after fluoxetine treatment (WT-CORT-flx: -29.0% *vs* WT-CORT, $p < 0.01$; KO-CORT-flx: -44.1% *vs* KO-CORT, $p < 0.001$) (Table 11).

In hippocampal areas, chronic RS67,333 treatment decreased *Arc* mRNA expression but restricted to the CA3 hippocampal field and, only in WT mice (WT-CORT-RS: -34.9% *vs* WT-CORT, $p < 0.05$) (Table 11 and Fig. 30C). Chronic treatment with fluoxetine was also associated with a downregulation of *Arc* mRNA expression in the CA1 and CA3 hippocampal fields, in both genotypes (WT-CORT-flx: -47.0% *vs* WT-CORT, $p < 0.001$; KO-CORT-flx: -52.7% *vs* KO-CORT: $p < 0.001$ for CA1; WT-CORT-flx: -36.5% *vs* WT-CORT, $p < 0.01$; KO-CORT-flx: -35.1% *vs* KO-CORT, $p < 0.01$ for CA3) (Table 12 and Fig. 30D).

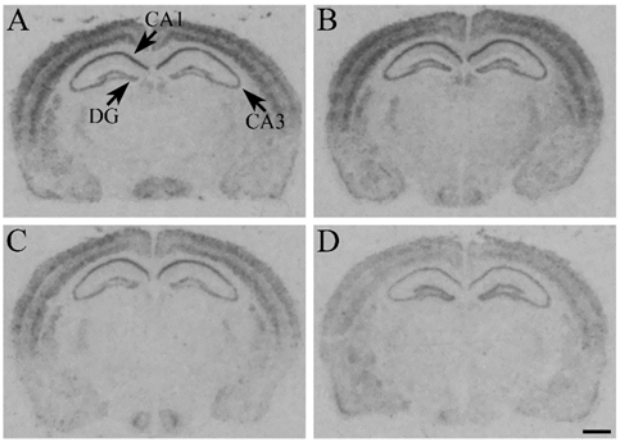


Figure 30. Autoradiograms showing the hippocampal *Arc* mRNA expression by *in situ* hybridization in the corticosterone model. WT-VEH (**A**), WT-CORT (**B**), WT-CORT-RS (**C**) and WT-CORT-flx (**D**). CA1 and CA3: CA1 and CA3 fields of the hippocampus and DG: dentate gyrus. Scale bar = 1 mm.

2.3.3.3. Effect of RS67,333 versus fluoxetine in non-CORT mice

A differential pattern, drug- and brain-region dependent, was also found in non-CORT mice administered RS67,333 compared to fluoxetine. Chronic treatment with RS67,333 regulated *Arc* mRNA expression in one hippocampal area (CA1 hippocampal field), whereas chronic fluoxetine treatment significantly changed its expression in almost all the brain structures analyzed (Tables 11 and 12).

In cortical areas, chronic fluoxetine did induce a great increase (≈ +60 %) in *Arc* mRNA expression on all the cortical areas in WT mice (Table 12). However, in KO mice, the SSRI reduced its expression in the medial prefrontal cortex and frontal cortex (Table 12).

In the hippocampus, chronic RS67,333 reduced the levels of *Arc* mRNA expression in the CA1 hippocampal field in WT mice (WT-RS: -35.6%, *vs* WT-VH, $p < 0.05$) but not in KO mice (Table 11). By contrast, chronic treatment with fluoxetine provoked a great increase (> +65) of *Arc* mRNA expression in CA1 and CA3 hippocampal fields in WT mice (WT-flx: +100.8% *vs* WT-VH, $p < 0.001$, for CA1; WT-flx: +67.6% *vs* WT-VH, $p < 0.01$ for CA3) but not in KO mice (Table 12).

Table 11. Effect of chronic RS67,333 treatment in *Arc* mRNA levels in the corticosterone model and non-CORT mice. Three-way ANOVA analyses revealed an interaction of genotype x treatment in CA3 hippocampal field ($F_{(1,49)}$: 10.1, $p < 0.01$). It also revealed a main effect of model in mPFrCx ($F_{(1,43)}$: 7.7, $p < 0.01$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, CA1: CA1 hippocampal field, CA3: CA3 hippocampal field, and DG: dentate gyrus. Data are mean ± SEM (nCi/g tissue) of n = 6-9 mice per group. $^{*}p < 0.05$ *vs* WT-VH, $^{+}p < 0.05$ *vs* WT-CORT, $^{\&}p < 0.05$ *vs* KO-VH (Newman-Keuls *post hoc* test).

	WT-VH	WT-RS	WT-CORT	WT-CORT-RS	KO-VH	KO-RS	KO-CORT	KO-CORT-RS
Cortical areas								
mPFrCx	21.1 ± 2.9	16.3 ± 3.2	19.5 ± 3.5	13.7 ± 2.2	20.7 ± 2,0	15.5 ± 1.4	**11.3 ± 0.7**$^{\&+}$	12.9 ± 1.8
FrCx	18.6 ± 2.9	17.0 ± 3.2	17.4 ± 3.0	14.7 ± 2.4	18.6 ± 1.8	14.3 ± 0.6	13.5 ± 2.4	13.8 ± 1.8
Cing Cx	29.4 ± 1.9	26.7 ± 5.4	25.9 ± 2.6	21.3 ± 1.5	25.3 ± 1.6	19.7 ± 2.8	22.6 ± 3.5	29.0 ± 3.3
Pyr Cx	16.1 ± 1.9	17.7 ± 2.7	16.3 ± 2.7	15.5 ± 2.4	15.4 ± 2	13.2 ± 1.8	11.4 ± 1.1	13.6 ± 2.2
Limbic areas								
Amygdala	7.0 ± 0.9	7.9 ± 1.9	8.2 ± 1.0	5.6 ± 1.5	6.3 ± 0.9	7.8 ± 1.0	6.2 ± 0.9	7.6 ± 1.1
Hippocampus								
CA1	46.2 ± 2.3	**29.7 ± 4.6***	37.3 ± 5.1	29.0 ± 4.6	41.2 ± 4.9	41.3 ± 5.2	34.6 ± 5.3	34.4 ± 5.9
CA3	20.5 ± 2.0	16.1 ± 2.3	23.0 ± 1.8	**15.0 ± 2.7**$^{+}$	19.3 ± 1.5	23.1 ± 2.1	17.9 ± 1.3	19.0 ± 2.0
DG	23.4 ± 4.5	25.3 ± 6.7	27.6 ± 4.6	25.5 ± 4.9	22.3 ± 4.7	31.7 ± 7.8	26.3 ± 6.6	23.7 ± 6.0

Table 12. Effects of chronic fluoxetine treatment in *Arc* mRNA levels in the corticosterone model and non-CORT mice. Three-way ANOVA analyses revealed an interaction effect of model x genotype x treatment in mPFrCx ($F_{(1,45)}$: 11.5, $p < 0.01$), FrCx ($F_{(1,45)}$: 15.5, $p < 0.001$), Cing Cx ($F_{(1,53)}$: 5.8, $p < 0.05$), Pyr Cx ($F_{(1,55)}$: 10.3, $p < 0.01$), CA1 ($F_{(1,57)}$: 9.9, $p < 0.01$) and CA3 ($F_{(1,57)}$: 5.4, $p < 0.05$). It also revealed an interaction effect of genotype x treatment in mPFrCx ($F_{(1,45)}$: 6.3, $p < 0.05$), FrCx ($F_{(1,45)}$: 11.7, $p < 0.01$), Cing Cx ($F_{(1,53)}$: 10.7, $p < 0.01$), Pyr Cx ($F_{(1,55)}$: 4.5, $p < 0.05$), CA1 ($F_{(1,57)}$: 9.1, $p < 0.01$) and CA3 ($F_{(1,57)}$: 4.1, $p < 0.05$). It also showed an interaction effect of model x treatment in Cing Cx ($F_{(1,53)}$: 21.7, $p < 0.001$), Pyr Cx ($F_{(1,55)}$: 11.8, $p < 0.01$), CA1 ($F_{(1,57)}$: 24.6, $p < 0.001$) and CA3 ($F_{(1,57)}$: 15.8, $p < 0.001$). A model x genotype interaction was found in Pyr Cx ($F_{(1,55)}$: 4.1, $p < 0.05$) and CA1 ($F_{(1,57)}$: 4.9, $p < 0.05$). It also revealed a main effect of model in mPFrCx ($F_{(1,45)}$: 33.0, $p < 0.001$), FrCx ($F_{(1,45)}$: 4.8, $p < 0.05$), Cing Cx ($F_{(1,53)}$: 34.6, $p < 0.001$), Pyr Cx ($F_{(1,55)}$: 21.0, $p < 0.001$), CA1 ($F_{(1,57)}$: 30.8, $p < 0.001$) and CA3 ($F_{(1,57)}$: 20.8, $p < 0.001$). A main effect of genotype in mPFrCx ($F_{(1,45)}$: 10.4, $p < 0.001$), Cing Cx ($F_{(1,53)}$: 5.0, $p < 0.05$), CA1 ($F_{(1,57)}$:14.2, $p < 0.001$), and CA3 ($F_{(1,57)}$: 5.3, $p < 0.05$). And a main effect of treatment in Pyr Cx ($F_{(1,55)}$: 7.4, $p < 0.01$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, CA1: CA1 hippocampal field, CA3: CA3 hippocampal field, and DG: dentate gyrus. Data are mean ± SEM (nCi/g tissue) of n = 6-9 mice per group. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* WT-VH, $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ *vs* WT-CORT, $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ *vs* KO-VH, and $^{€€}p < 0.01$ and $^{€€€}p < 0.001$ *vs* KO-CORT (Newman-Keuls *post hoc* test).

	WT-VH	WT-flx	WT-CORT	WT-CORT-flx	KO-VH	KO-flx	KO-CORT	KO-CORT-flx
Cortical areas								
mPFrCx	21.7 ± 1.4	**35.4 ± 8.2****	17.6 ± 1.4	**11.4 ± 1.3**$^{+}$	23.9 ± 2.5	**15.5 ± 1.6**$^{\&}$	**12.7 ± 2.1**$^{\&\&}$	10.7 ± 2.1
FrCx	14.2 ± 0.9	**25.0 ± 2.8*****	16.3 ± 2.8	12.1 ± 0.9	20.1 ± 1.5	**11.1 ± 1.6**$^{\&\&}$	16.5 ± 2.4	13.8 ± 2.0
Cing Cx	30.1 ± 1.4	**56.8 ± 13.2****	30.1 ± 1.9	**21.3 ± 2.2**$^{++}$	35.7 ± 2.3	33.9 ± 5.3	29.6 ± 3.3	**16.5 ± 0.8**$^{€€€}$
Pyr Cx	17.1 ± 1.5	**46.5 ± 10.2*****	17.3 ± 2.2	12.7 ± 1.5	24.0 ± 1.9	26.2 ± 7.4	18.1 ± 3.0	19.0 ± 4.0
Limbic areas								
Amygdala	8.6 ± 1.3	10.3 ± 1.7	8.3 ± 1.1	8.1 ± 1.3	9.7 ± 0.8	7.3 ± 1.4	8.3 ± 1.4	8.9 ± 1.8
Hippocampus								
CA1	44.5 ± 2.9	**89.4 ± 14.4*****	45.9 ± 3.9	**24.4 ± 2.3**$^{+++}$	45.3 ± 3.2	39.6 ± 1.0	39.0 ± 5.0	**18.5 ± 1.5**$^{€€€}$
CA3	24.4 ± 2.3	**40.9 ± 6.4****	25.3 ± 2.2	**16.0 ± 1.9**$^{++}$	25.9 ± 1.7	24.6 ± 3.9	22.7 ± 1.5	**14.7 ± 1.7**$^{€€}$
DG	22.5 ± 3.0	26.5 ± 4.7	18.2 ± 2.5	24.3 ± 5.3	22.5 ± 3.2	20.5 ± 5.7	20.0 ± 2.5	16.1 ± 4.0

2.4. Molecular studies: western blot of mTOR and β-catenin and their phosphorylated forms

After chronic corticosterone treatment, mTOR levels and p-mTOR/mTOR ratio in the hippocampus of both genotypes did not differ from those detected under non-CORT conditions (Figs. 31A and B). Regarding hippocampal β-catenin protein levels, we found an increase in corticosterone-treated WT mice (WT-CORT: 56.7% *vs* WT-VH, $p < 0.05$), and a trend to an increase in corticosterone-treated KO mice (KO-CORT: 36.3% *vs* KO-VH) (Fig. 31C). Nevertheless, the ratio p-β-catenin/β-catenin was unaltered after corticosterone treatment in both genotypes (Fig. 31D).With regards to the effect of chronic RS67,333, no changes were found in the protein levels of mTOR and β-catenin and their phosphorylated forms in CORT and non-CORT mice, in both genotypes (Fig. 31).

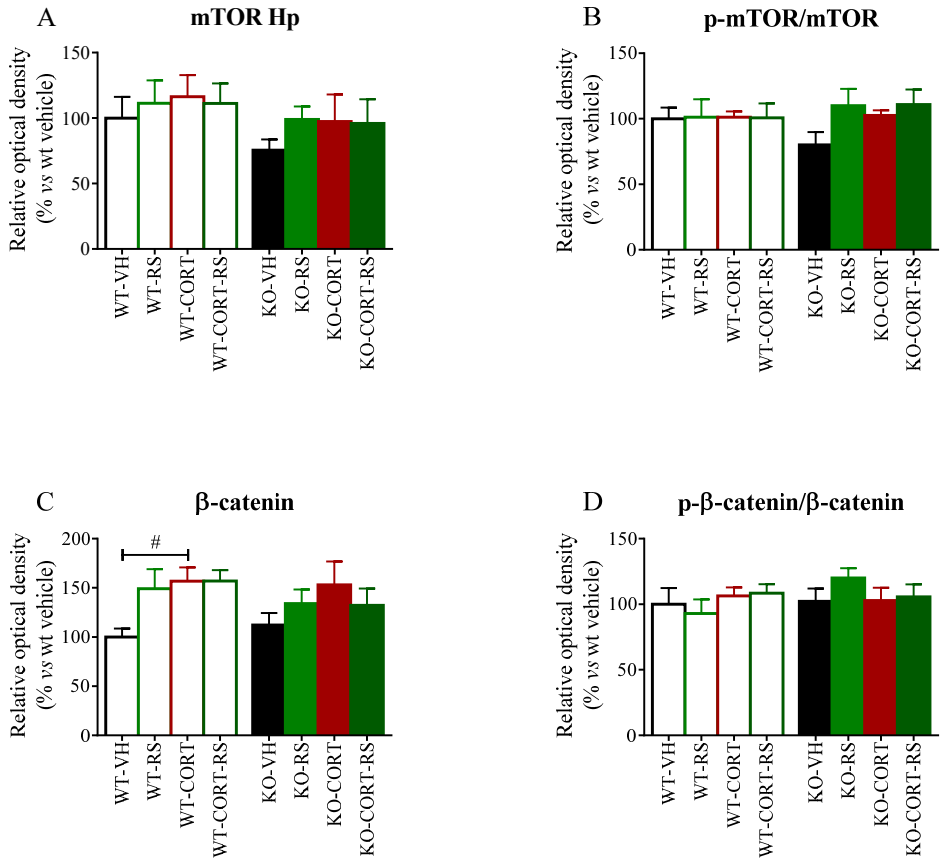


Figure 31. Effect of chronic RS67,333 treatment for 14 days on mTOR, p-mTOR/mTOR, β-catenin and p-β-catenin/β-catenin in the hippocampus in the corticosterone model and non-CORT mice. Data are mean ± SEM of *n* =: 6-9 mice per group. $^{\#}p < 0.05$ (Student´s *t*-test, unpaired data).

3. OBX MODEL OF DEPRESSION: EFFECT OF RS67,333 AND FLUOXETINE

Bilateral olfactory bulbectomy (OBX) is one rodent model of depression/anxiety owning a high predictive validity since OBX-induced manifestations are reversed by clinically used antidepressants (Song and Leonard, 2005); although its construct validity may be questioned. However, it has been proposed to resemble a neurodegenerative-like model with relevant alterations on the serotonergic neurotransmission and neuroplasticity-related pathways (Hendriksen *et al*., 2015).

As described for the corticosterone model, we analysed the behavioural, molecular and neurochemical consequences of the loss of the 5-HT_4 receptors in the development and manifestations of the OBX syndrome. Given the predictive properties of this model, we compared the pharmacological actions of RS67,333 with those of the reference antidepressant fluoxetine with the aim of evaluating the onset and characteristics of its antidepressant/anxiolytic actions.

3.1. Behavioural studies

3.1.1. OBX model

The olfactory bulbectomy surgery induced locomotor hyperactivity in mice of both genotypes, a characteristic feature of the model, when they were tested at four weeks post-surgery under a novelty arena with high luminance conditions. The total distance travelled was increased substantially, in a similar magnitude, in WT-OBX and KO-OBX mice (+66.5% and +73.9% *vs* sham-operated counterparts, $p < 0.001$ and $p < 0.01$, respectively) (Fig. 32A). This locomotor hyperactivity was related to an enhanced thigmotaxis as evidenced by the increased peripheral ambulation in both WT-OBX (+96.9 % *vs* WT-sham, $p < 0.001$) and KO-OBX (+82.6 *vs* OBX-sham, $p < 0.001$) mice (Fig. 32B).

Regarding anxiety parameters, mice of both genotypes showed a similar OBX-induced anxiety-like behavior in the open field (Figs. 32C and D). They exhibited lower number of central entries (WT-OBX: -61.5% *vs* WT-sham; KO-OBX: -51.3% *vs* OBX-sham, $p < 0.001$for both comparison) (Fig. 32D), and lower central time,

although the effect of the surgery upon the central time parameter was not so noticeable in KO-OBX mice (WT-OBX: -63.7 % *vs* WT-sham, $p < 0.001$; KO-OBX: -29.4% *vs* KO-sham, $p = 0.09$ one-tailed unpaired *t*-test) due to the lower value they already had before the surgery (Fig. 32C).

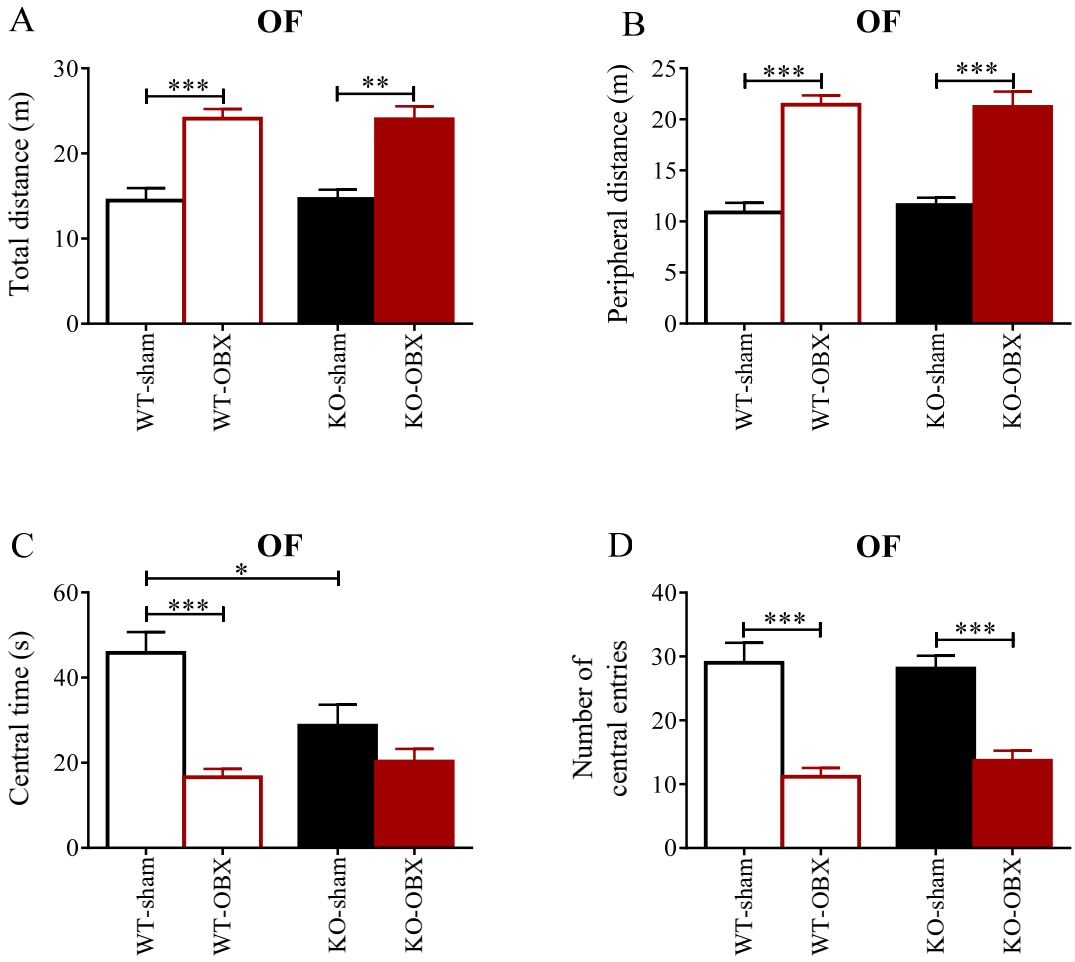


Figure 32. Effect of olfactory bulbectomy on the hyperactivity and the anxiety levels in the open field. Total distance travelled (**A**), peripheral distance (**B**) central time (**C**), and number of central entries (**D**) were measured 4 weeks after olfactory bulbectomy surgery in the OF test. Two-way ANOVA analyses revealed an interaction effect of genotype x model on the central time ($F_{(1,53)}$: 7.0, $p < 0.05$). Moreover, two-way ANOVA analyses revealed a main effect of model on the total distance travelled ($F_{(1,53)}$: 32.4, $p < 0.001$), peripheral distance ($F_{(1,53)}$: 46.4, $p < 0.001$), central time ($F_{(1,53)}$: 19.3, $p < 0.001$) and number of entries ($F_{(1,53)}$: 61.7, $p < 0.001$). Data are mean ± SEM of n = 14-15 mice per group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Newman-Keuls *post hoc* test).

3.1.2. Effect of RS67,333 in the OBX model

Once the OBX-induced behavior was confirmed, WT and KO mice were chronically treated with RS67,333 (1.5 mg/kg/day, once daily), and tested in the open field at different time-points (7, 14 and 28 days post-treatment). As shown in figures 33A and B, chronic treatment with RS67,333, at all the time points, did not attenuate OBX-induced locomotor hyperactivity (Fig. 33A) and neither the enhanced thigmotaxis (augmented activity in the periphery, Fig. 33B).

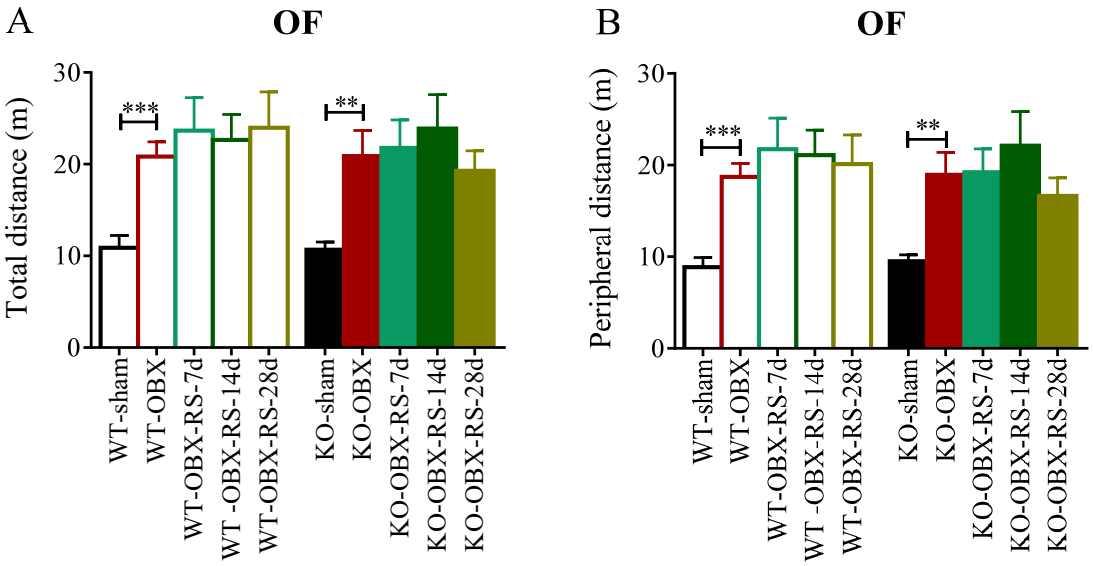


Figure 33. Effect of chronic administration of RS67,333 in OBX mice on locomotor activity and peripheral activity in the open field. Total distance travelled (**A**) and peripheral distance (**B**) were measured after 7, 14 and 28 days of treatment with RS67,333 in the OF. Two-way ANOVA analyses revealed a main effect of time on the total distance travelled ($F_{(4,81)}$: 5.4, $p < 0.001$) and peripheral distance ($F_{(4,81)}$: 5.9, $p < 0.001$. Data are mean ± SEM of n = 9-10 mice per group. $^{**}p < 0.01$ and $^{***}p < 0.01$ (Newman-Keuls *post hoc* test).

However, its sustained administration resulted in increased central activity in WT-OBX mice, as evidenced by a higher number of central entries (WT-OBX-RS: +97.6 % *vs* WT-OBX, $p < 0.05$, after 28 days of treatment, Fig. 34B) and a trend towards increased values of central time (Fig. 34A). Both findings were absent in KO-OBX counterparts treated with the 5-HT_4 partial agonist (Figs. 34A and B). Therefore, and in a similarly to the model of corticosterone, chronic treatment with RS67,333 exhibited an anxiolytic effect in the OBX model in WT mice but not in KO mice.

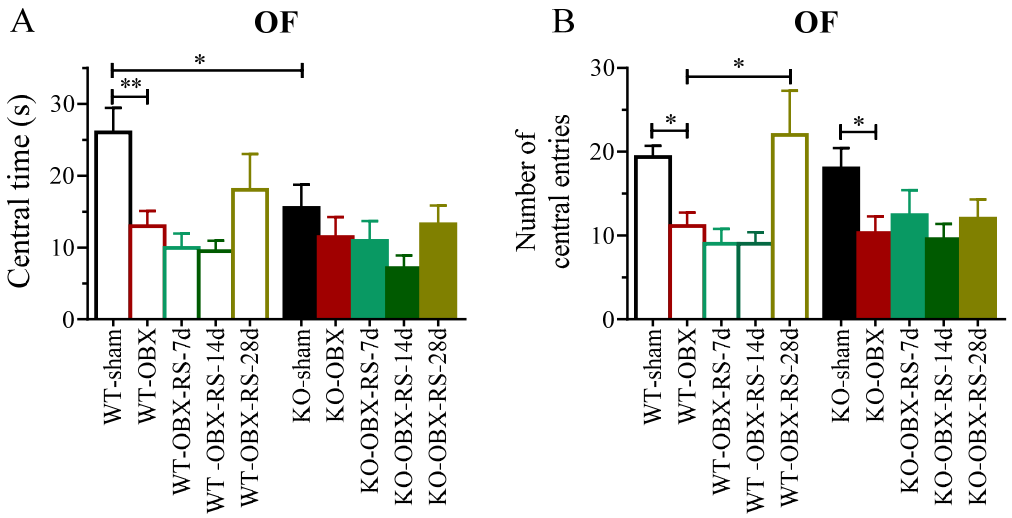


Figure 34. Effect of chronic administration of RS67,333 in OBX mice the central activity in the open field. Central time (**A**) and number of central entries (**B**) were measured after 7, 14 and 28 days of treatment with RS67,333 in the OF. Two-way ANOVA analyses revealed a main effect of central time ($F_{(4,81)}$: 6.1, $p < 0.001$) and number of central entries ($F_{(4,81)}$: 6.1, $p < 0.001$). Additionally, two-way ANOVA showed a main effect of genotype on the central time ($F_{(1,81)}$: 4.6, $p < 0.05$) Data are mean ± SEM of n = 9-10 mice per group. *$p < 0.05$ and **$p < 0.01$ (Newman-Keuls *post hoc* test).

Given the lack of effect of the chronic treatment with RS67,333 upon the OBX-induced hyperactivity and anxiety, we wondered whether the 5-HT_4 partial agonist would be able to modify other emotional features. Therefore, in a separate set of animals, we evaluated the effect of RS67,333 in the sucrose intake test. As previously indicated, KO-sham mice presented a significant reduction in the sucrose intake respect to WT counterparts ($p < 0.01$). Bulbectomized mice from both genotypes presented a significant decrease in sucrose intake (WT-OBX: 0.9 ± 0.1 ml and KO-OBX: 1.0 ± 0.1 ml, $p < 0.001$ for both genotypes *vs* respective sham group). Furthermore, and unlike to that observed in the corticosterone model, chronic treatment with RS67,333 was unable to counteracts OBX-induced anhedonia in both genotype (Fig. 35).

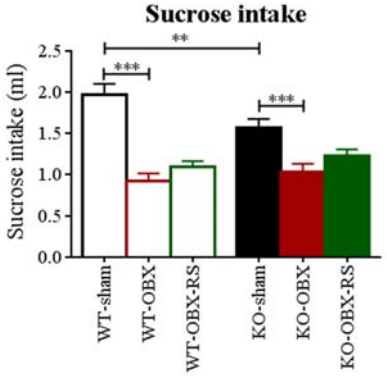


Figure 35. Effect of chronic administration of RS67,333 in OBX mice in the sucrose intake. Two-way ANOVA analyses revealed a treatment x genotype interaction after 14 days of treatment with RS67,333 on sucrose intake ($F_{(2,48)}$: 4.7, $p < 0.05$). A main effect of treatment on sucrose intake was found ($F_{(2,48)}$: 33.7, $p < 0.001$). Data are mean ± SEM of n = 8-10 mice per group. **$p < 0.01$ and ***$p < 0.001$ (Newman-Keuls *post hoc* test).

3.1.3. Effect of fluoxetine in the OBX model

Chronic treatment with fluoxetine induced a significant reversion of the OBX-induced hyperactivity in WT mice, but not in KO mice. In fact, after 7 days of treatment, a significant reversion of OBX-induced changes in the total (WT- OBX: 25.2 ± 2.8 m *vs* WT-OBX-flx-7d: 18.7 ± 4.1 m, $p < 0.05$) (Fig. 36A) and peripheral (WT-OBX: 23.4 ± 2.7 m *vs* WT-OBX-flx-7d: 17.2 ± 3.9 m, $p < 0.05$) (Fig. 36B) distances were observed.

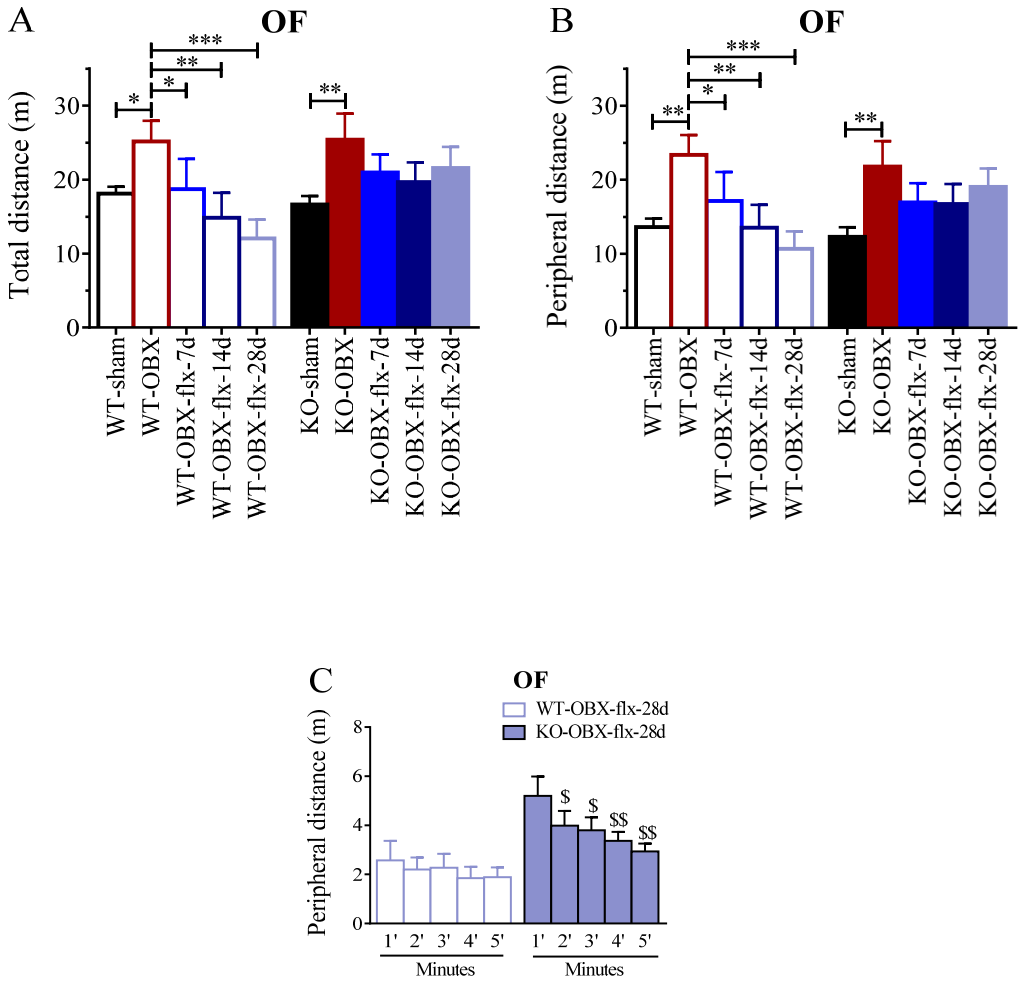


Figure 36. Effect of chronic administration of fluoxetine in OBX mice in the locomotor activity in the open field. Total distance travelled (**A**) and peripheral distance (**B**) were evaluated before and following fluoxetine (flx) treatment. Additionally, peripheral distance per one min intervals at day 28 of fluoxetine treatment is represented (**C**). Two-way ANOVA revealed a genotype x time interaction on the distance travelled at the periphery ($F_{(4,48)}$: 2.7, $p < 0.05$) and total distance ($F_{(4,48)}$: 2.8, $p < 0.05$). Two-way ANOVA also revealed a main effect of treatment on peripheral distance ($F_{(4,48)}$: 2.7, $p < 0.05$) and total distance ($F_{(4,48)}$: 7.5, $p < 0.001$). In the case of peripheral distance per one min intervals at day 28 of fluoxetine treatment (flx 28d), the two-way ANOVA revealed an effect of time ($F_{(4,52)}$: 4.7, $p < 0.01$) and genotype ($F_{(1,13)}$: 6.6, $p < 0.05$). Data are mean ± SEM of n = 7-8 mice per group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ *vs* 1 min intervals (Newman-Keuls *post hoc* test).

This effect was maintained after 14 and 28 days of treatment (Figs. 36A and B). Therefore, chronic fluoxetine treatment reduced, in a time-dependent manner, the characteristic OBX induced locomotor hyperactivity in WT mice (7 days: -91.3% and 63.6% for total and peripheral distance, respectively; 14 days: -145.9 % and -100.7 % for total and peripheral distance; and 28 days: -185.2 % and -130.1% for total and peripheral distance) (Figs. 36A and B). As mentioned above, chronic administration of fluoxetine failed to reverse OBX-induced hyperactivity in 5-HT$_4$ KO mice [achieving only ~15% of reduction in the total distance travelled and peripheral distance travelled, (Figs. 36A and B)]. Additionally, as shown in Fig. 36C, chronic administration of fluoxetine in 5-HT$_4$ KO mice failed to elicit the beneficial effect regarding the habituation pattern to a novel/aversive environment (high luminance-open field), which was observed in WT mice.

The administration of fluoxetine did not modify the OBX-induced anxiety behaviour in both genotypes at any time-point, as evidenced by the lack of changes in both the central time and the number of entries (Figs. 37A and B).

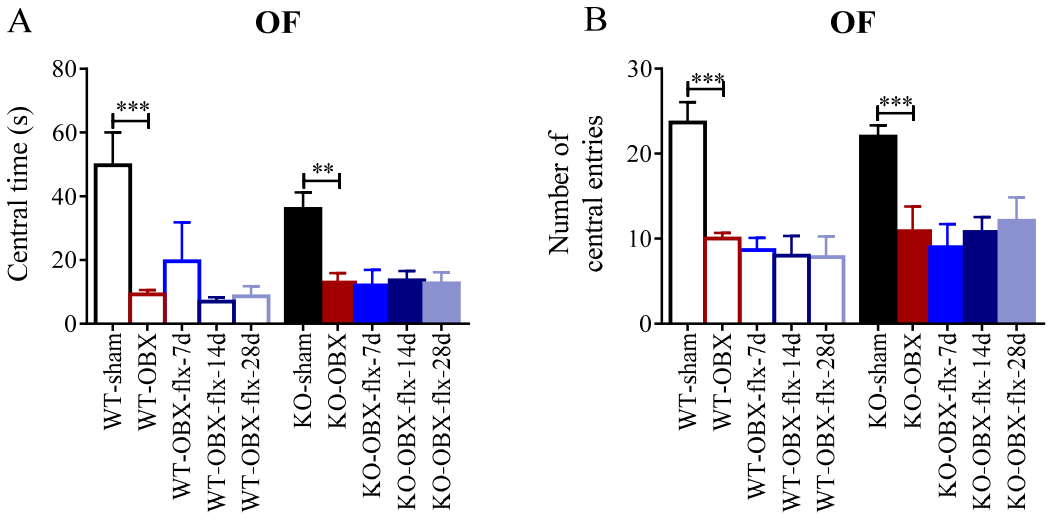


Figure 37. Effect of chronic administration of fluoxetine in OBX mice in the central activity in the open field. Central time (**A**) and number of entries (**B**) were evaluated before and following fluoxetine (flx) treatment.Two-way ANOVA revealed a main effect of treatment on central time ($F_{(4,52)}$: 18.9, $p < 0.001$) and number of entries ($F_{(4,52)}$: 18.5, $p < 0.001$). Data are mean ± SEM of n = 7-8 mice per group. $^{**}p < 0.01$ and $^{***}p < 0.05$ (Newman-Keuls *post hoc* test).

Regarding the effect of chronic fluoxetine in depression-related tests, it was not feasible to carry them due to the low number of KO and WT subjects available, which were assigned to the studies assessing the effects of RS67,333 in this behavioural paradigm. Nevertheless, data from our group demonstrated the anti-anhedonic effect of fluoxetine after chronic administration in the OBX model in C57BL/6 (Vidal *et al*., 2013).

3.2. Neurochemical studies: 5-HT$_{1A}$ receptor functionality

Using [^{35}S]GTPγS binding autoradiography in brain sections, we evaluated whether the OBX model and/or the chronic treatment with RS67,333 and fluoxetine could regulate 5-HT$_{1A}$ receptors functionality. Here below, we are describing the effects of both drugs in mice subjected to the OBX model.

Regarding the findings on the effects of both drugs in non-OBX (sham) mice, we did assume the results obtained in non-CORT mice (as a comparable experimental group) in order to minimize and optimize the number of available subjects.

3.2.1. OBX model

In the **OBX model**, we analyzed the functionality of 5-HT$_{1A}$ receptors across the brain, and, as shown in Tables 13, 14 and figure 38 olfactory bulbectomy increase the functionality of the 5-HT$_{1A}$ receptor in the DRN of KO mice and the CA3 hippocampal field of both genotypes (Tables 13, 14). Olfactory bulbectomy did not modify the specific basal [^{35}S]GTPγS binding across the brain (data no shown).

In addition, KO-sham mice displayed a desensitization of 5-HT$_{1A}$ receptors in the DRN compared to WT-sham littermates. Also, a significant increase in basal [^{35}S]GTPγS binding values was detected in the DRN in 5-HT$_4$ KO mice compared with their counterparts (WT-sham= 391.8 ± 17.3 *vs* KO-sham= 445.9 ± 9.5 nCi/g tissue).

3.2.2. Effect of RS67,333 *versus* fluoxetine in the OBX model

A region-, genotype- and drug-dependent regulation of 5-HT_{1A} receptors functionality in OBX mice was observed after the chronic administration of RS67,333 and fluoxetine.

In the midbrain, a significant reduction of the functionality of 5-HT_{1A} receptors was detected after the chronic treatment with fluoxetine in the DRN in OBX mice of both genotypes. This 5-HT_{1A} autoreceptor desensitization was higher in WT-OBX-flx mice (WT-OBX-flx: 2.7% *vs* WT-OBX: 39.2%, $p < 0.001$) (Table 14 and Fig. 38D) than in KO-OBX-flx mice (KO-OBX-flx: 13.6% *vs* KO-OBX: 34.5%, $p < 0.05$) (Table 14). Regarding the MRN, a significant reduction of the 5-HT_{1A} functionality was found only in WT-OBX mice treated chronically with fluoxetine (WT-OBX-flx: -4.7% *vs* WT-OBX: 20.2, $p < 0.05$) (Table 14 and Fig. 38D). Chronic administration of RS67,333 did not modify 5-HT_{1A} autoreceptor functionality in WT OBX mice and reverted the effects of surgery 5-HT_{1A} receptor functionality in KO mice (KO-OBX-RS: 32.3% *vs* KO-OBX: 44.4%, $p < 0.05$) (Table 13).

In cortical areas, the functionality of 5-HT_{1A} receptors remained unchanged after the chronic administration of RS67,333 or fluoxetine (Tables 13 and 14).

In the hippocampus, chronic fluoxetine had no effect in WT-OBX mice whereas it reduced the % stimulation of [^{35}S] GTPγS binding induced by (±)-8-OH-DPAT in KO mice in both layers of the CA1 field (KO-OBX-flx: 41.2% *vs* KO-OBX: 75.2%, $p < 0.05$ for CA1 oriens; KO-OBX-flx: 59.2% *vs* KO-OBX: 101.5%, $p < 0.05$ for CA1 radiatum) (Table 14). Regarding the effect of RS67,333, a reduction in the 5-HT_{1A} receptor functionality was observed in the CA3 hippocampal field respect to non-treated WT- and KO-OBX mice (WT-OBX-RS: 19.8% *vs* WT-OBX: 46.3%, $p < 0.05$; KO-OBX-RS: 5.4% *vs* KO-OBX: 45.3%, $p < 0.001$) (Table 13).

Table 13. Effect of chronic RS67,333 treatment on the % stimulation of specific [^{35}S]GTPγS induced by (±)-8-OH-DPAT in the OBX model. Two-way ANOVA analyses revealed a main effect of genotype in DRN ($F_{(1,46)}$: 5.7, p < 0.05), Ent Cx ($F_{(1,40)}$: 5.1, $p < 0.05$), and a main effect of treatment in Ent Cx ($F_{(2,40)}$: 3.2, $p < 0.05$) and CA3 ($F_{(2,40)}$: 12.48, $p < 0.001$). DRN: dorsal raphe nucleus, MRN: median raphe nucleus, Ent Cx: entorhinal cortex, mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, CA1 oriens: CA1 oriens hippocampal field, CA1 rad: CA1 radiatum hippocampal field, CA3: CA3 hippocampal field, and DG: dentate gyrus. Data are mean ± SEM of *n* = 7-9 mice per group and values are expressed as percentage of (±)-8-OH-DPAT stimulated [^{35}S]GTPγS binding. $^{*}p < 0.05$ *vs* WT-sham, $^{+}p < 0.05$ *vs* WT-OBX, $^{\&}p < 0.05$ *vs* KO-VH, $^{€}p < 0.05$ and $^{€€€}p < 0.001$ *vs* KO-OBX (Newman-Keuls *post hoc* test) and $^{\#}p < 0.05$ *vs* WT-sham (Student´s *t*-test, unpaired data).

	WT-sham	WT-OBX	WT-OBX-RS	KO-sham	KO-OBX	KO-OBX-RS
Brainstem areas						
DRN	50.8 ± 8.9	43.4 ± 9.4	44.1 ± 5.6	**27.0 ± 4.9**$^{\#}$	**44.4 ± 4.6**$^{\&}$	**32.3 ± 3.5**$^{€}$
MRN	17.6 ± 7.2	25.5 ± 5.1	20.0 ± 5.8	10.8 ± 10.4	29.6 ± 6.0	20.3 ± 5.8
Cortical areas						
mPFrCx	47.9 ± 8.9	32.7 ± 11.6	49.0 ± 20.3	37.1 ± 9.2	44.0 ± 12.3	36.1 ± 14.0
FrCx	57.2 ± 7.8	36.7 ± 12.5	32.5 ± 9.9	44.4 ± 6.0	43.9 ± 5.9	32.9 ± 10.1
Ent Cx	46.2 ± 5.8	64.7 ± 12.2	62.2 ± 11.3	28.3 ± 6.4$^{\#}$	55.5 ± 8.5	40.2 ± 4.7
Hippocampal areas						
CA1 oriens	80.4 ± 11.6	101.8 ± 14.5	100.7 ± 15.8	110.5 ± 6.6	90.9 ± 15.5	81.6 ± 16.4
CA1 rad	105.1 ± 9.7	112.3 ± 16.8	118.9 ± 18.1	133.8 ± 12.5	106.7 ± 17.1	105.6 ± 14.9
CA3	18.7 ± 3.8	**46.3 ± 10.8***	**19.8 ± 6.7**$^{+}$	21.3 ± 5.3	**45.3 ± 11.8**$^{\&}$	**5.4 ± 3.9**$^{€€€}$
DG	26.5 ± 5.4	29.9 ± 4.3	19.0 ± 4.9	27.8 ± 5.3	27.9 ± 5.1	26.4 ± 5.5

Table 14: Effect of chronic fluoxetine treatment on the % stimulation of specific [^{35}S]GTPγS induced by (±)-8-OH-DPAT in the OBX model. Two-way ANOVA revealed an interaction genotype x treatment in DRN ($F_{(2,51)}$: 4.6, $p < 0.05$). It also revealed a main effect of treatment in DRN ($F_{(2,51)}$: 12.3, $p < 0.001$), MRN ($F_{(2,54)}$: 7.7, p < 0.01), FrCx ($F_{(2,39)}$: 3.5, $p < 0.01$), CA1 oriens ($F_{(2,43)}$: 5.3, $p < 0.01$) and CA1 rad ($F_{(2,43)}$: 17.1, $p < 0.01$). A main effect of genotype in FrCx ($F_{(1,39)}$: 9.2, $p < 0.01$), CA1 oriens ($F_{(1,43)}$: 6.5, p < 0.05) and CA1 rad ($F_{(1,43)}$: 15.2, $p < 0.001$) was also detected. DRN: dorsal raphe nucleus, MRN: median raphe nucleus, Ent Cx: entorhinal cortex, mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, CA1 oriens: CA1 oriens hippocampal field, CA1 rad: CA1 radiatum hippocampal field, CA3: CA3 hippocampal field, and DG dentate gyrus. Data are mean ± SEM of *n* = 6-10 mice per group. $^{**}p < 0.01$ *vs* WT-sham, ^{+}p <0.05 and ^{+++}p <0.001 *vs* WT-OBX, $^{\&}p < 0.05$ *vs* KO-sham and $^{€}p < 0.05$ vs KO-OBX (Newman-Keuls *post hoc* test).

	WT-sham	WT-OBX	WT-OBX-flx	KO-sham	KO-OBX	KO-OBX-flx
Brainstem areas						
DRN	44.1 ± 8.4	39.2 ± 3.6	**2.7 ± 5.3**$^{+++}$	**19.3 ± 5.2****	**34.5 ± 5.1**$^{\&}$	**13.6 ± 6.2**$^{€}$
MRN	19.0 ± 5.5	20.2 ± 3.6	**-4.7 ± 6.5**$^{+}$	16.9 ± 4.8	21.0 ± 4.8	**7.3 ± 5.9**
Cortical areas						
mPFrCx	61.1 ± 8.9	90.1 ± 29.8	56.2 ± 25.5	40.8 ± 8.9	48.7 ± 11.9	13.7 ± 13.7
FrCx	101.7 ± 19.8	70.1 ± 14.7	69.5 ± 12.5	64.5 ± 8.5	52.3 ± 11.1	39.3 ± 13.5
Ent Cx	58.5 ± 21.2	37.6 ± 7.6	27.1 ± 8.8	44.0 ± 11.0	18.7 ± 8.3	24.7 ± 3.4
Hippocampal areas						
CA1 oriens	105.6 ± 18.1	96.3 ± 10.4	67.1 ± 16.2	78.8 ± 10.2	75.2 ± 9.7	**41.2 ± 9.9**$^{€}$
CA1 rad	139.4 ± 17.7	129.0 ± 6.8	103.2 ± 22.6	101.9 ± 9.3	101.5 ± 8.9	**59.2 ± 8.5**$^{€}$
CA3	20.1 ± 7.3	**47.3 ± 6.4****	48.3 ± 14.8	19.0 ± 6.1	**40.5 ± 5.4**$^{\&}$	25.1 ± 9.9
DG	48.6 ± 8.4	50.1 ± 9.6	36.8 ± 15.1	29.2 ± 6.7	41.5 ± 8.2	30.9 ± 9.7

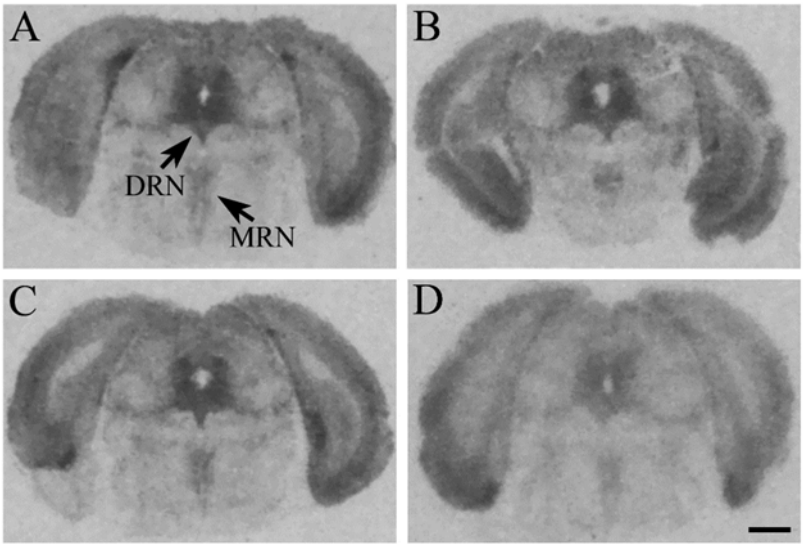


Figure 38. **Representative autoradiograms of (±)-8-OH-DPAT stimulated [^{35}S]GTPγS binding in the olfactory bulbectomy model at the level of midbrain**. WT-Sham (**A**), WT-OBX (**B**), WT-OBX-RS (**C**) and WT-OBX-flx (**D**). DRN: dorsal raphe nucleus, MRN: median raphe nucleus. Scale bar = 1 mm.

3.3. Molecular studies: mRNA expression of neuroplasticity markers

In situ hybridization studies were carried out using coronal brain sections obtained from WT and KO mice. Our aim was to evaluate whether the OBX model and/or the chronic treatment with RS67,333 and fluoxetine modulated the levels of the mRNA expression of BDNF/trkB and *Arc*. Herein, we are describing the effects of both drugs in mice subjected to the OBX model.

As for the drug effects in WT- and KO-sham mice, and in order to minimize and optimize the number of available subjects, we are using, for comparative purposes, the results obtained in non-CORT, an experimental group exposed to a similar handling.

3.3.1. BDNF mRNA expression

3.3.1.1. OBX model

In cortical areas, the OBX syndrome did not modify BDNF mRNA expression in the mPFrCx and FrCx regions (Figs 39A and B); however, reduced levels were detected in the cingulate cortex (WT-OBX: -21.8% *vs* WT-sham, $p < 0.05$; KO-OBX: -30.7% *vs* KO-sham, $p < 0.001$) (Fig. 39C), whereas increased levels were measured in the pyriform cortex (WT-OBX: ≈ + 57.1% *vs* WT-sham, $p < 0.001$; KO-OBX: ≈ +48.8% *vs* KO-sham, $p < 0.01$) (Figs. 39D and 42A).

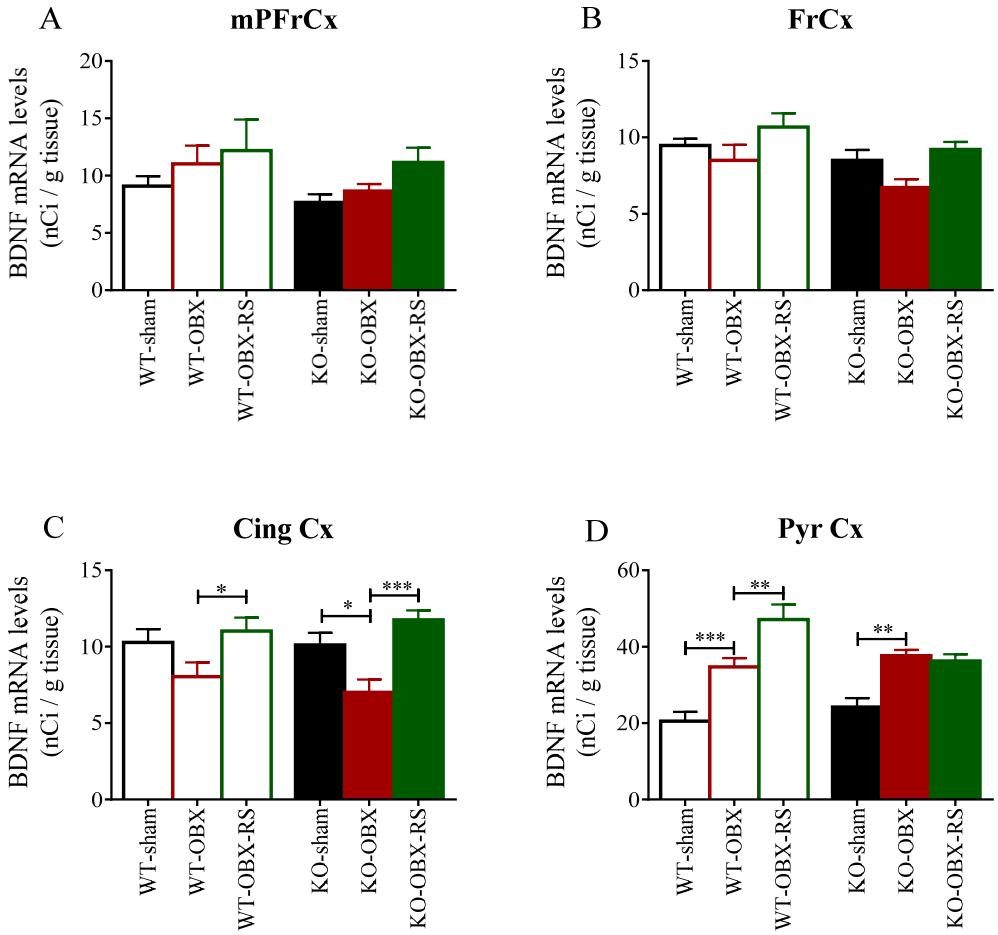


Figure 39. Effects of chronic RS67,333 treatment in BDNF mRNA levels in several cortical areas in WT- and KO-OBX mice. Two-way ANOVA analyses revealed an interaction of genotype x treatment in pyriform cortex ($F_{(2,45)}$: 4.8, $p < 0.05$), and a main effect of treatment in cingulate cortex ($F_{(2,42)}$: 11.6, $p < 0.001$) and pyriform cortex ($F_{(2,45)}$: 27.4, $p < 0.001$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex. Data are mean ± SEM of n = 8-10 mice per group. *$p < 0.05$ and **$p < 0.01$, ***$p < 0.001$ (Newman-Keuls *post hoc* test).

In the hippocampus, BDNF mRNA expression in WT- and KO-mice was not modified by OBX (Figs. 40, 41B and 42B-D), although KO-OBX animals exhibited lower levels of mRNA expression in CA3 field (KO-OBX: ≈-20%, $p < 0.05$, *vs* WT-OBX, (Figs. 40B, 41B and 42C).

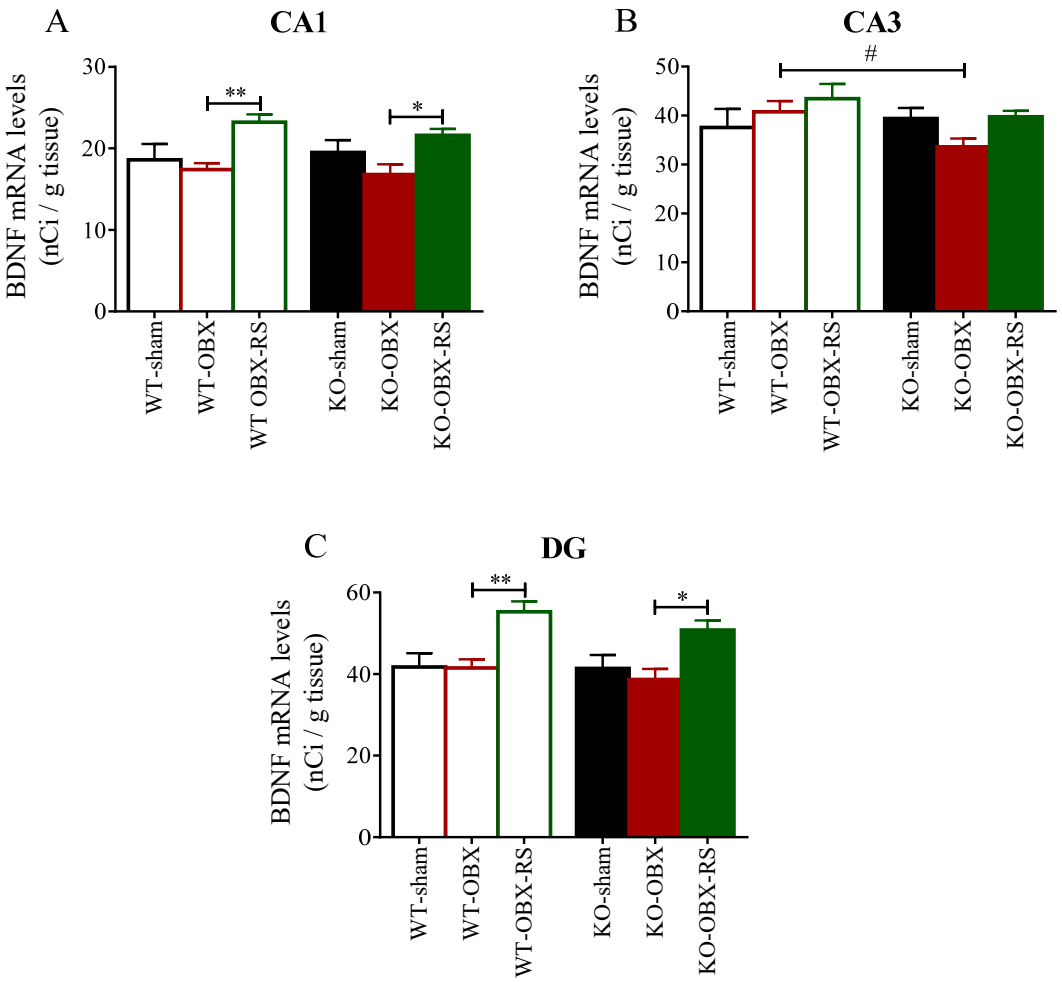


Figure 40. Effects of chronic RS67,333 treatment in BDNF mRNA levels in the hippocampus in WT- and KO-OBX mice. Two-way ANOVA analyses revealed a main effect of treatment CA1 ($F_{(2,49)}$: 9.0, $p < 0.001$) and DG ($F_{(2,50)}$: 9.7, $p < 0.001$). CA1: CA1 field of the hippocampus, CA3: CA3 field of the hippocampus, DG: dentate gyrus of the hippocampus. Data are mean ± SEM of n = 8-10 mice per group $^{*}p < 0.05$ and $^{**}p < 0.01$ (Newman-Keuls *post hoc* test), $^{\#}p < 0.05$ (Student´s *t* test, unpaired data).

3.3.1.2. Effect of RS67,333 versus fluoxetine in the OBX model

Chronic treatment with RS67,333 increased the levels of expression of BDNF mRNA, whereas chronic fluoxetine treatment, especially in WT-OBX mice, induced a reduction in the BDNF mRNA expression in some cortical and hippocampal areas.

In cortical areas, a trend towards increased expression was observed in the mPFrCx and FrCx, in bulbectomized mice of both genotypes receiving chronic RS67,333 (Figs. 39A and B). In the cingulate cortex, it did significantly increased BDNF mRNA expression in both genotypes (WT-OBX-RS: +37.3% *vs* WT-OBX, $p < 0.05$; KO-OBX-RS: +67.5% *vs* KO-OBX, $p < 0.01$) (Fig. 39C). In the pyriform cortex, chronic RS67,333 also induced an upregulation of BDNF mRNA expression, but only in WT-OBX mice (WT-OBX-RS: +35.6% *vs* WT-OBX, $p < 0.01$) (Fig. 39D).

Chronic treatment with fluoxetine reversed, only in WT mice, the OBX-induced changes in the levels of BDNF mRNA expression in the pyriform cortex (WT-OBX-flx: -47.3 *vs* WT-OBX, $p < 0.01$) (Fig. 42A).

In the hippocampus, chronic RS67,333 increased the levels of BDNF mRNA expression (around + 30%), in both genotypes, in the CA1 hippocampal field (WT-OBX-RS: +33,3% *vs* WT-OBX, $p < 0.01$; KO-OBX-RS: +28.7% *vs* KO-OBX, $p < 0.05$) (Fig. 40A and 41C) and the DG field (WT-OBX-RS: +33.2% *vs* WT-OBX, $p < 0.01$; KO-OBX-RS: +31.5% *vs* KO-OBX, $p < 0.05$) (Figs. 40C and 41C).

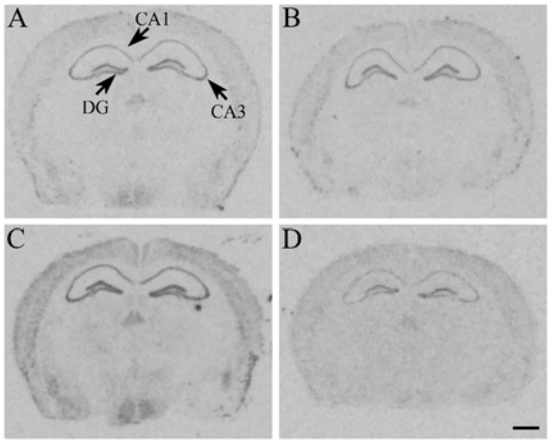


Figure 41. Autoradiograms showing the hippocampal BDNF mRNA expression by *in situ* hybridization in the olfactory bulbectomy model. WT- Sham (**A**), WT-OBX (**B**), WT-OBX-RS (**C**) and WT-OBX-flx (**D**). CA1 and CA3: CA1 and CA3 fields of the hippocampus and DG: dentate gyrus. Scale bar = 1 mm.

Regarding the effects of chronic fluoxetine treatment, a significant downregulation of the levels of BDNF mRNA expression was observed in the CA1 hippocampal field, in both genotypes (WT-OBX-flx: -36.4% *vs* WT-OBX, $p < 0.01$; KO-OBX-flx: -35.4% *vs* KO-OBX, $p < 0.01$) (Figs. 41D and 42B). In other hippocampal areas, significant changes were detected in WT mice but not KO mice (WT-OBX-flx: -38.6% *vs* WT-OBX, $p < 0.01$ for CA3 field, and WT-OBX-flx: -27.4% *vs* WT-OBX, $p < 0.01$ for DG) (Figs. 41D, 42C and D).

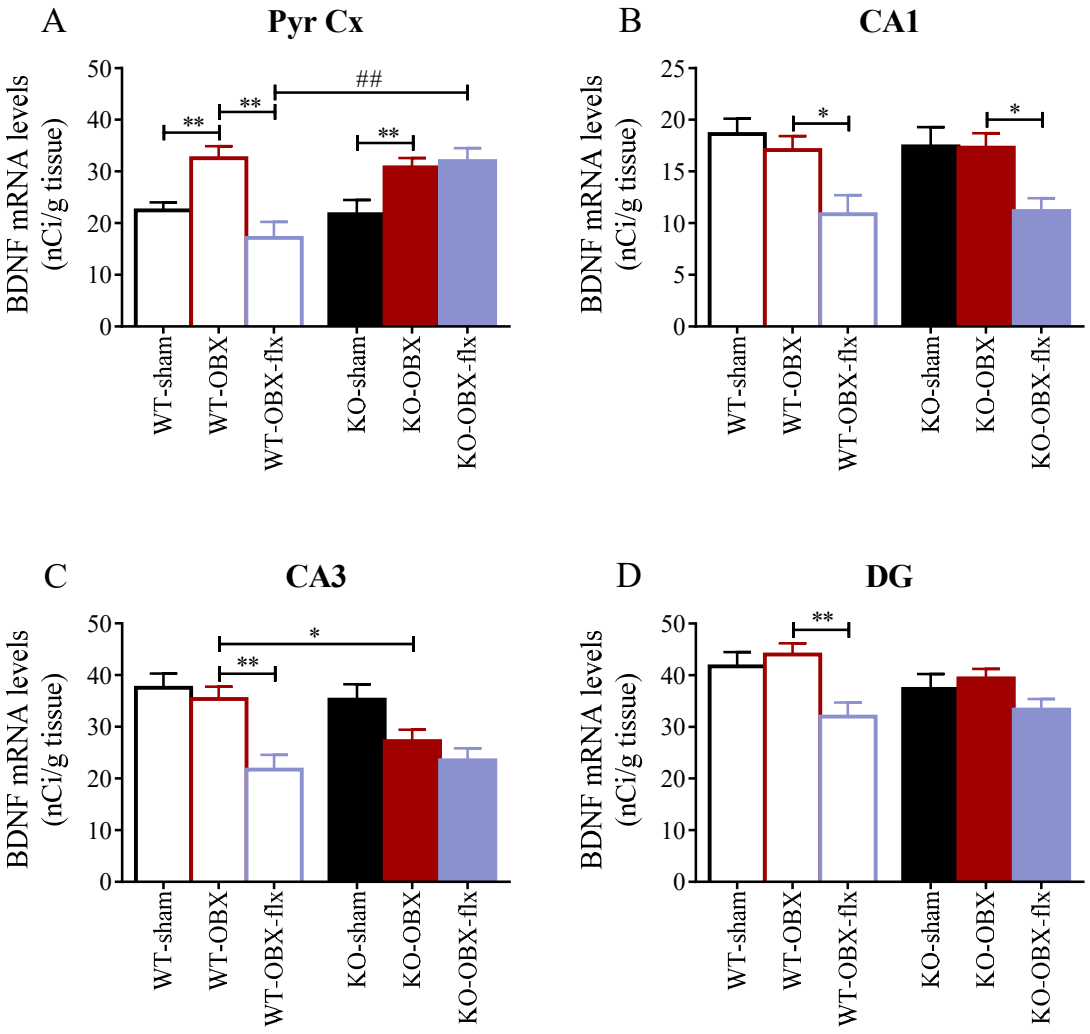


Figure 42. Effects of chronic fluoxetine treatment in BDNF mRNA levels in cortico-hippocampal areas in WT- and KO-OBX mice. Two-way ANOVA analyses revealed an interaction of genotype x treatment in pyriform cortex ($F_{(2,46)}$: 5.6, $p < 0.01$). It also show a main effect of treatment in pyriform cortex ($F_{(2,46)}$: 9.9, $p < 0.001$), CA1 ($F_{(2,57)}$: 7.0, $p < 0.001$), CA3 ($F_{(2,57)}$: 11.7, $p < 0.001$) and DG ($F_{(2,55)}$: 4.9, $p < 0.05$). A main effect of genotype in the pyriform cortex ($F_{(1,46)}$: 4.1, $p < 0.05$) was also revealed. Pyr Cx: pyriform cortex, CA1: CA1 field of the hippocampus, CA3: CA3 field of the hippocampus, DG: dentate gyrus of the hippocampus. Data are mean ± SEM of n = 8-11 mice per group. *$p < 0.05$ and **$p < 0.01$ (Newman-Keuls *post hoc* test), $^{\#\#}p < 0.01$ (Student´s *t* test, unpaired data).

A correlation analysis was performed plotting the levels of BDNF mRNA expression in the dentate gyrus against the values of the total distances in the OF, merging all the individual values of all the experimental groups of figures 40C and 42D. The total distance correlated with the levels of BDNF mRNA ($r^2 = 0.126$, $p < 0.01$) (Fig. 43A). Moreover, as shown in figures 43B and C, we further analyzed this correlation in the groups of OBX-mice. A significant correlation was found in WT-OBX mice treated with fluoxetine or its vehicle (r^2: 0.559, $p < 0.01$) (Fig. 43C) but not in the case of WT-CORT mice treated with RS67, 333 or its vehicle (r^2: 0.05 ns) (Fig 43B).

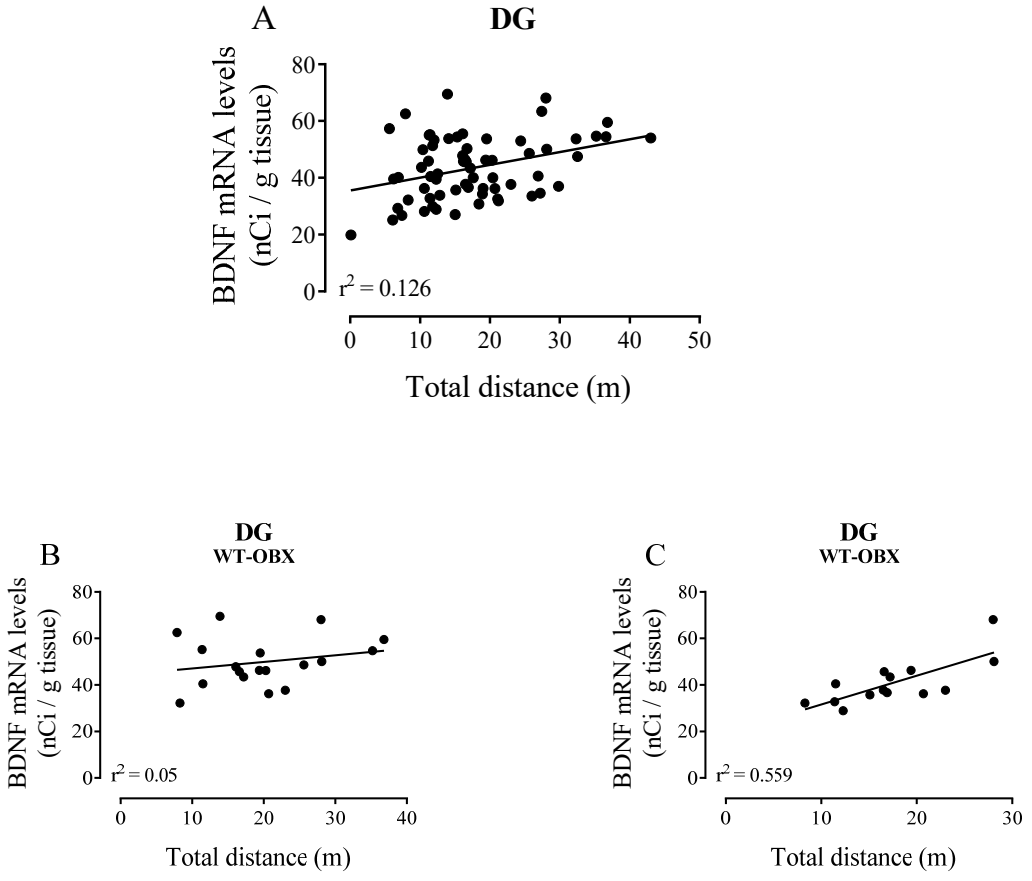


Figure 43. **Correlation between BDNF mRNA expression in the dentate gyrus and open field locomotor activity readouts.** The regression lines for BDNF mRNA expression *versus* the total distance in the OF. Plotting all the experimental groups (**A**), WT-OBX mice treated with RS67,333 or its vehicle (**B**) and WT-OBX mice treated with fluoxetine or its vehicle(**C**). Note the direct relationship between BDNF mRNA expression and the locomotor activity (the higher expression of BDNF, the higher locomotor activity) in fluoxetine treated WT-OBX mice but not in mice treated with RS67,333.

3.3.2. TrkB mRNA expression

3.3.2.1. OBX model

Increased levels of trkB mRNA expression were detected in the nucleus accumbens in both WT-OBX (≈ 40.0% *vs* WT-sham, $p < 0.05$) and KO-OBX (≈ 41.5% *vs* KO-sham, $p < 0.01$) mice (Tables 15 and 16). No changes were observed in other brain areas.

3.3.2.2. Effect of RS67,333 versus fluoxetine in the OBX model

Both drugs exerted a similar effect in OBX mice in a genotype-dependent manner. Chronic RS67,333 reversed the increased expression observed in the nucleus accumbens in WT-OBX (WT-OBX-RS: -30.2% *vs* WT-OBX, $p < 0.05$) but not in KO-OBX mice (Table 15). However, RS67,333 reduced trkB mRNA expression in the pyriform cortex in KO-OBX mice (KO-OBX-RS, -32.1% *vs* WT-OBX, $p < 0.01$) (Table 15).

Table 15. Effects of chronic RS67,333 treatment in TrkB mRNA levels in WT- and KO-OBX mice. Two-way ANOVA analyses revealed an interaction genotype x treatment in pyriform cortex ($F_{(2,43)}$: 3.5, $p < 0.05$), and a main effect of treatment in pyriform cortex ($F_{(2,43)}$: 3.5, $p < 0.05$), nucleus accumbens ($F_{(2,41)}$: 9.0, $p < 0.001$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, Nuc acc: nucleus accumbens, CA1: CA1 field of the hippocampus, CA3: CA3 field of the hippocampus, DG: dentate gyrus of the hippocampus. Data are mean ± SEM (nCi/g tissue) of n = 7-10 mice per group. $^{**}p < 0.01$ *vs* WT-sham, $^{+}p < 0.05$ *vs* WT-OBX, $^{\&\&}p < 0.01$ *vs* KO-sham, $^{\epsilon\epsilon}p < 0.01$ *vs* KO-OBX (Newman-Keuls *post hoc* test).

	WT-sham	WT-OBX	WT-OBX-RS	KO-sham	KO-OBX	KO-OBX-RS
Cortical areas						
mPFrCx	33.6 ± 2.2	39.8 ± 3.9	37.8 ± 6.0	36.3 ± 2.4	43.3 ± 5.5	38.4 ± 4.5
FrCx	25.3 ± 2.2	31.5 ± 2.3	29.7 ± 3.8	29.4 ± 1.3	31.0 ± 4.6	28.7 ± 4.0
Cing Cx	35.9 ± 1.6	38.0 ± 2.7	34.0 ± 3.2	36.0 ± 1.9	41.7 ± 4.0	36.5 ± 4.0
Pyr Cx	56.4 ± 4.0	56.4 ± 4.3	53.4 ± 6.0	56.3 ± 3.7	67.9 ± 3.4	**46.1 ± 4.7**$^{\epsilon\epsilon}$
Limbic areas						
Nuc acc	24.0 ± 1.1	**34.9 ± 3.4****	**24.4 ± 2.7**$^{+}$	22.5 ± 1.2	**31.5 ± 3.8**$^{\&\&}$	26.0 ± 2.5
Amygdala	85.2 ± 4.7	74.8 ± 4.6	68.6 ± 6.2	87.9 ± 6.3	80.8 ± 6.1	65.0 ± 6.8
Hypothalamus	104.9 ± 5.8	103.1 ± 6.1	87.4 ± 7.9	113.8 ± 8.6	98.9 ± 3.7	78.8 ± 7.9
Hippocampus						
CA1	86.5 ± 5.0	83.7 ± 3.6	66.8 ± 7.6	92.4 ± 6.7	84.4 ± 3.5	68.9 ± 6.4
CA3	91.3 ± 4.4	85.8 ± 3.9	69.5 ± 6.7	100.5 ± 6.9	81.8 ± 3.9	67.6 ± 6.1
DG	87.9 ± 4.4	92.4 ± 4.9	74.6 ± 7.7	95.4 ± 7.9	96.9 ± 3.8	79.0 ± 7.3

Chronic treatment with fluoxetine decreased trkB mRNA expression in the nucleus accumbens in WT-OBX (WT-OBX-flx: -37.6% *vs* WT-OBX, $p < 0.05$) but not in KO-OBX mice (Table 16).

Chronic treatment with RS67,333 and fluoxetine did not modify trkB mRNA expression levels in the other brain areas analyzed (Tables 15 and 16).

Table 16. Effects of chronic fluoxetie treatment in TrkB mRNA levels in WT- and KO-OBX mice. Two-way ANOVA analyses revealed a main effect of treatment in the nucleus accumbens ($F_{(2,52)}$: 14.3, $p < 0.001$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, Nuc acc: nucleus accumbens, CA1: CA1 field of the hippocampus, CA3: CA3 field of the hippocampus, DG: dentate gyrus of the hippocampus. Data are mean ± SEM (nCi/g tissue) of n = 7-10. $^{**}p < 0.01$ *vs* WT-sham; $^{+}p < 0.05$ *vs* WT-OBX, $^{\&}p < 0.05$ *vs* KO-sham (Newman-Keuls *post hoc* test).

	WT-sham	WT-OBX	WT-OBX-flx	KO-sham	KO-OBX	KO-OBX-flx
Cortical areas						
mPFrCx	66.2 ± 5.0	65.8 ± 5.4	62.4 ± 7.7	60.4 ± 4.3	57.6 ± 5.1	70.9 ± 8.9
FrCx	53.4 ± 3.7	53.9 ± 4.7	52.7 ± 4.5	51.7 ± 3.7	45.7 ± 4.7	57.9 ± 5.3
Cing Cx	81.1 ± 2.7	79.0 ± 2.8	65.0 ± 6.3	72.3 ± 5.1	81.6 ± 5.4	67.6 ± 4.8
Pyr Cx	123.8 ± 6.4	119.2 ± 7.1	93.9 ± 10.4	113.0 ± 7.8	119.0 ± 9.3	101.0 ± 5.9
Limbic areas						
Nuc acc	46.9 ± 1.5	**63.2 ± 5.1****	**39.5 ± 4.4**$^{+}$	41.2 ± 2.3	**58.9 ± 5.4**$^{\&}$	50.8 ± 3.1
Amygdala	66.1 ± 2.3	60.4 ± 4.4	59.7 ± 5.6	57.3 ± 3.1	58.2 ± 3.0	58.5 ± 3.7
Hypothalamus	89.8 ± 5.2	79.3 ± 4.4	90.2 ± 6.3	84.8 ± 5.2	73.1 ± 5.0	90.0 ± 6.5
Hippocampus						
CA1	74.6 ± 4.2	71.2 ± 4.1	72.5 ± 2.6	71.0 ± 2.6	72.2 ± 4.5	76.2 ± 3.7
CA3	71.8 ± 4.6	68.5 ± 3.8	72.1 ± 5.2	73.2 ± 4.3	68.0 ± 5.3	71.9 ± 4.4
DG	77.0 ± 4.7	76.3 ± 4.8	79.0 ± 6.6	76.8 ± 3.2	74.4 ± 4.9	78.2 ± 4.7

3.3.3. *Arc* mRNA expression

3.3.3.1. OBX model

OBX surgery did not modify the *Arc* mRNA levels observed in the brain of the both genotypes (Tables 17 and 18).

3.3.3.2. Effect of RS67,333 versus fluoxetine in the OBX model

In cortical areas, chronic administration of fluoxetine, but not RS67,333 reduced the *Arc* mRNA expression. This downregulation was more apparent in WT-OBX than in KO-OBX mice. Indeed, in WT-OBX mice, chronic fluoxetine decreased *Arc*

mRNA expression in the mPFrCx and FrCx (WT-OBX-flx: -60.9% *vs* WT-OBX, $p < 0.05$ for mPFrCx; WT-OBX-flx: -60.1 % *vs* WT-OBX, $p < 0.01$ for FrCx) (Table 18) and a tendency towards reduction was found in the pyriform cortex (WT-OBX-flx: -41.7 % vs WT-OBX, $p < 0.05$). Nevertheless, a downregulation of *Arc* mRNA expression in the cingulate cortex was detected in both WT and KO-OBX mice (WT-OBX-flx: -60.0 % *vs* WT-OBX, $p < 0.05$; KO-OBX-flx: -37.6 % *vs* KO-OBX, $p < 0.05$) (Table 18). In the amygdala, neither chronic RS67,333 nor fluoxetine modulated *Arc* mRNA expression after olfactory bulbectomy in both genotypes.

Table 17. Effects of chronic RS67333 treatment in *Arc* mRNA levels in WT- and KO-OBX mice. Two-way ANOVA analyses revealed a main effect of treatment ($F_{(2,46)}$: 8.2, $p < 0.001$) and genotype ($F_{(2,46)}$: 4.2, $p < 0.05$) in the DG. mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, CA1: CA1 field of the hippocampus, CA3: CA3 field of the hippocampus, DG: dentate gyrus of the hippocampus. Data are mean ± SEM (nCi/g tissue) of n = 8-10 mice per group. $^{++}p < 0.01$ *vs* WT-OBX (Newman-Keuls *post hoc* test).

	WT-sham	WT-OBX	WT-OBX-RS	KO-sham	KO-OBX	KO-OBX-RS
Cortical areas						
mPFrCx	14.6 ± 3.5	19.9 ± 5.4	19.2 ± 4.1	11.1 ± 2.5	14.6 ± 1.6	18.7 ± 5.7
FrCx	13.4 ± 3.9	16.4 ± 2.8	18.1 ± 3.4	14.1 ± 4.1	14.8 ± 1.2	18.8 ± 4.1
Cing Cx	33.6 ± 6.9	19.9 ± 4.0	25.4 ± 5.2	26.6 ± 5.9	23.7 ± 2.3	24.3 ± 4.4
Pyr Cx	7.7 ± 1.2	11.2 ± 1.4	15.7 ± 2.1	14.2 ± 2.1	13.2 ± 2.2	14.9 ± 1.8
Limbic areas						
Amygdala	3.8 ± 0.8	4.4 ± 1.0	6.6 ± 2.0	4.8 ± 1.0	4.2 ± 0.6	6.2 ± 1.0
Hippocampus						
CA1	40.7 ± 8.3	56.8 ± 12.9	52.8 ± 9.6	38.8 ± 5.3	45.7 ± 3.0	37.6 ± 7.9
CA3	18.3 ± 3.2	26.5 ± 5.7	28.6 ± 4.9	18.4 ± 2.3	21.7 ± 1.5	21.9 ± 3.5
DG	9.0 ± 1.6	18.3 ± 5.1	**33.5 ± 6.7**$^{++}$	11.3 ± 1.9	13.9 ± 2.2	17.1 ± 2.0

In the hippocampus, a great increase in the levels of *Arc* mRNA expression was found in the DG in WT-OBX mice chronically treated with RS67,333 (WT-OBX-RS: +83.1% *vs* WT-OBX, $p < 0.01$), and effect that was not present in KO-OBX counterparts (Table 17and Fig. 44C). Regarding the effects of fluoxetine, a similar pattern to that observed in cortical areas was found in the hippocampal fields. Indeed, in CA1 field fluoxetine caused a downregulation of *Arc* mRNA expression only in WT-OBX mice (WT-OBX-flx: -48.8% *vs* WT-OBX, $p < 0.05$) (Table 18 and Fig. 44D). However, fluoxetine treatment was associated with downregulated *Arc* mRNA expression, in both genotypes of OBX, in CA3 field (WT-OBX-flx: -44.7% *vs* WT-OBX, $p < 0.05$; KO-OBX-flx: -25.1% *vs* KO-OBX, $p < 0.05$) (Table

18 and Fig. 44D) and DG (WT-OBX-flx: -55.3% *vs* WT-OBX, $p < 0.01$; KO-OBX-flx: -44.8% *vs* KO-OBX, $p < 0.01$) (Table 18 and Fig. 44D).

Table 18. Effects of chronic fluoxetine treatment in *Arc* mRNA levels in WT- and KO-OBX mice. Two-way ANOVA analyses revealed a main effect of treatment in the mPFrCx ($F_{(2,44)}$: 7.4, $p < 0.01$), FrCx ($F_{(2,43)}$: 7.8, $p < 0.01$), cingulate cortex ($F_{(2,52)}$: 11.1, $p < 0.001$), CA1 hippocampal field ($F_{(2,55)}$: 6.2, $p < 0.01$), CA3 hippocampal field ($F_{(2,55)}$: 5.5, $p < 0.01$) and DG ($F_{(2,53)}$: 3.8, $p < 0.05$), and a main effect of genotype in the mPFrCx ($F_{(1,44)}$: 4.3, $p < 0.05$). mPFrCx: medial prefrontal cortex, FrCx: frontal cortex, Cing Cx: cingulate cortex, Pyr Cx: pyriform cortex, CA1: CA1 field of the hippocampus, CA3: CA3 field of the hippocampus, DG: dentate gyrus of the hippocampus. Data are mean ± SEM (nCi/g tissue) of n = 7-11 mice per group. $^{+}p < 0.05$ and $^{++}p < 0.01$ *vs* WT-OBX, $^{€}p < 0.05$ and $^{€€}p < 0.01$ *vs* KO-OBX (Newman-Keuls *post hoc* test); $^{\#}p < 0.05$ *vs* WT-OBX (Student´s *t* test, unpaired data).

	WT-sham	WT-OBX	WT-OBX-flx	KO-sham	KO-OBX	KO-OBX-flx
Cortical areas						
mPFrCx	21.7 ± 3.6	27.3 ± 5.2	**10.7 ± 3.0**$^{+}$	14.0 ± 1.9	19.4 ± 2.1	10.0 ± 2.4
FrCx	17.5 ± 2.7	23.3 ± 3.5	**9.3 ± 3.4**$^{++}$	12.8 ± 2.0	18.2 ± 1.7	12.4 ± 1.7
Cing Cx	31.0 ± 1.1	23.3 ± 3.5	**9.3 ± 1.9**$^{+}$	24.5 ± 3.0	25.5 ± 2.3	**15.9 ± 1.8**$^{€}$
Pyr Cx	14.6 ± 3.0	12.7 ± 1.7	**7.4 ± 0.8**$^{\#}$	17.9 ± 3.5	15.3 ± 3.0	11.8 ± 1.8
Limbic areas						
Amygdala	8.5 ± 1.1	6.3 ± 1.1	4.2 ± 1.5	4.6 ± 0.8	4.8 ± 0.9	3.6 ± 0.9
Hippocampus						
CA1	45.8 ± 4.3	45.1 ± 7.2	**23.1 ± 5.7**$^{+}$	34.9 ± 3.7	39.3 ± 2.6	29.9 ± 2.1
CA3	24.2 ± 3.2	19.9 ± 2.4	**11.0 ± 2.8**$^{+}$	18.0 ± 2.5	18.5 ± 0.9	**13.9 ± 1.0**$^{€}$
DG	21.4 ± 4.3	19.9 ± 2.0	**8.9 ± 3.0**$^{++}$	18.7 ± 4.1	17.4 ± 2.2	**9.6 ± 1.4**$^{€€}$

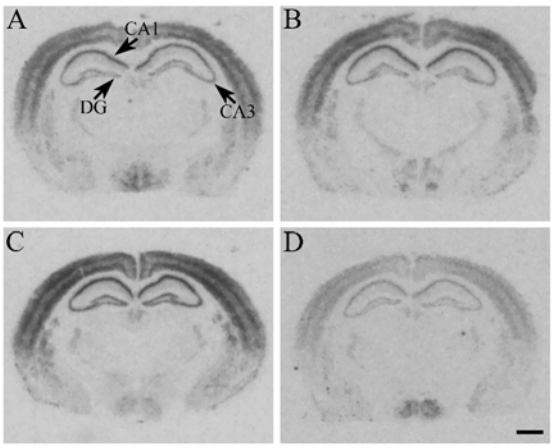


Figure 44. Autoradiograms showing the hippocampal *Arc* mRNA expression by *in situ* hybridization in the olfactory bulbectomy model. WT- Sham (**A**), WT-OBX (**B**), WT-OBX-RS (**C**) and WT-OBX-flx (**D**).). CA1 and CA3: CA1 and CA3 fields of the hippocampus and DG: dentate gyrus. Scale bar = 1 mm.

3.4. Molecular studies: western blot of mTOR and β-catenin and their phosphorylated forms

Following olfactory bulbectomy, mTOR levels were decreased in WT (WT-OBX: -42.6% *vs* WT-sham, $p < 0.05$) but not KO mice (KO-OBX: -19.6% *vs* WT-sham) (Fig. 45A); however, the p-mTOR/mTOR ratio in the hippocampus of both genotypes did not differ from those detected in sham counterparts (Fig. 45B). After 28-day treatment with RS67,333, a total reversion of the effects of the OBX surgery on the mTOR protein levels in WT-OBX mice (WT-OBX: +80.3% *vs* WT-OBX-RS, $p < 0.05$) was found (Fig. 45A). Nevertheless, chronic RS67,333 did not modify the p-mTOR / mTOR ratio in OBX mice of both genotypes (Fig. 45B).

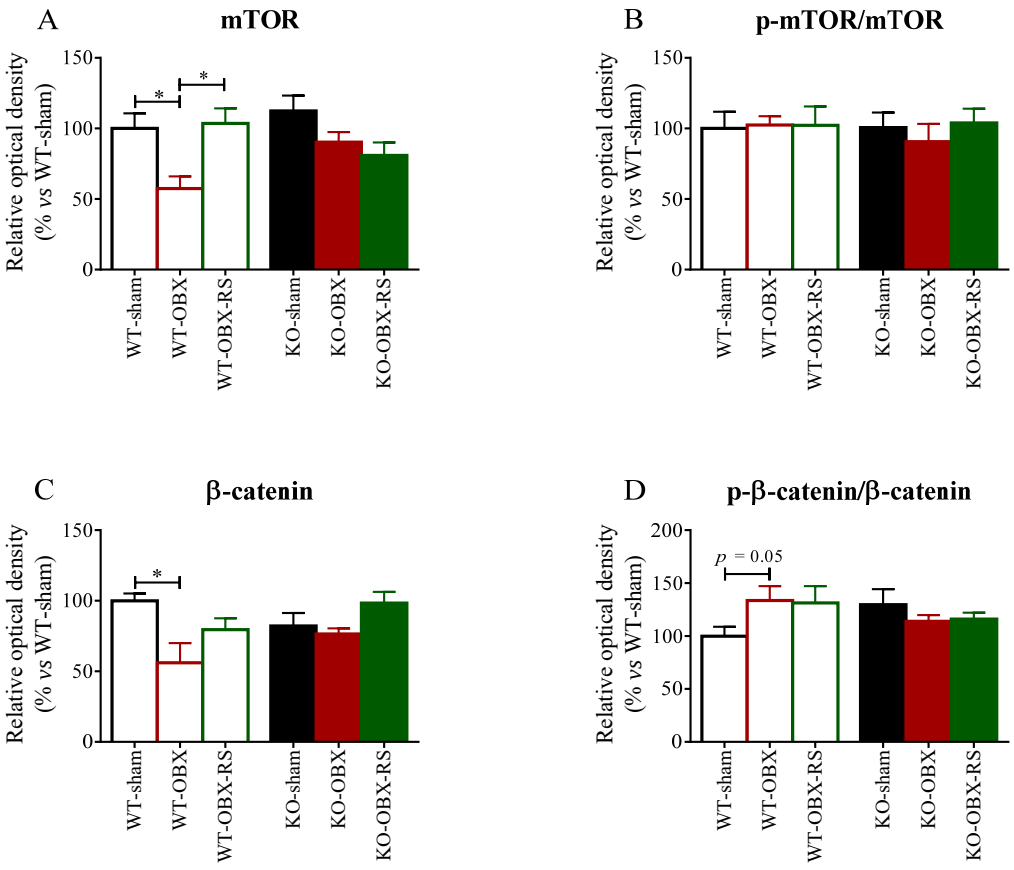


Figure 45. Effects of chronic RS67,333 treatment in mTOR, p-mTOR/mTOR, β-catenin and p-β-catenin/β-catenin in the hippocampus of OBX mice. Two-way ANOVA analyses revealed a interaction effect of genotype x treatment on the levels of mTOR ($F_{(2,41)}$: 3.6, $p < 0.05$) and on the levels of β-catenin ($F_{(2,37)}$: 3.9, $p < 0.05$). Moreover, two-way ANOVA analyses also revealed a main effect of the treatment on the levels of mTOR ($F_{(2,41)}$: 4.5, $p < 0.05$) and β-catenin ($F_{(2,37)}$: 4.9, $p < 0.05$). Data are mean ± SEM of n = 7-8 mice per group. *$p < 0.05$ (Newman-Keuls *post hoc* test).

Regarding hippocampal β-catenin protein levels, we found a decrease in WT-OBX mice but not in KO-OBX mice (WT-OBX: -44.0% vs WT-sham, $p < 0.05$, Fig. 45C). Moreover, WT-OBX mice presented a trend towards increased values of p-β-catenin/β-catenin ratio (WT-OBX: +33.6% vs WT-sham, $p = 0.05$, Fig. 45D). Chronic RS67,333 treatment did no modify β-catenin levels (Fig. 45C) and the p-β-catenin/β-catenin ratio in OBX mice of both genotypes (Fig. 45D).

"*En cuestiones de ciencia, la autoridad de miles no vale más que el humilde razonamiento de un único individuo.*"

Galileo Galilei

V. Discussion

1. BEHAVIOURAL CONSEQUENCES OF THE 5-HT$_4$ RECEPTOR DELETION

5-HT$_4$ KO mice show innate context-dependent anxiety and anhedonia

We first evaluated whether the genetic deletion of 5-HT$_4$ receptor could influence the "basal" **anxiety** (innate or anxiety trait) by using different paradigms (see summarizing figure 46). In ethological-type tests (approach-avoidance conflict), namely open field and light/dark box tests, we have found differential behavioural outcomes. On one hand, 5-HT$_4$ KO mice spent less time in the center of the open field, suggesting an increased anxiety-like response, in concordance with Compan *et al.* (2004) but not with Kobayashi *et al*. (2011). In the light/dark box, our KO mice behaved similarly to WT counterparts as reported by Kobayashi *et al*. (2011). The anxious-like phenotype that we observe in the 5-HT$_4$ KO mice is in good agreement with the behavioural results we have obtained in C57BL/6 mice, which demonstrate that the acute administration of the 5-HT$_4$ agonist RS67,333 induced a dose-dependent anxiolytic effect in the open field test (Castro *et al.,* 2014b).

It is worth to mention that both the experimental conditions and the mouse strain used may influence the behavioural outcomes. We have tested the mice under a highly illuminated arena (i.e. 400 luxes in the open field) whereas in Kobayashi´s study they were tested under low-luminance (40 luxes) conditions. It is well known that a highly illuminated open field may induce latent anxiety (Gould *et al.,* 2009 libro). Therefore, such aversive contextual environment might have unveiled an innate anxiety ("trait") that our KO mice could have. Anyway, we cannot discard the influence of the genetic background of the mutant mice used in different studies (C57BL/6 in the Kobayashi´s study and sv129 in ours); this is an important issue regarding anxiety-related behaviours and the sensitivity to anxiolytic drugs (Griebel *et al.,* 2000; Van Gaalen and Steckler, 2000). Moreover, as explained below, not all the tests may be reflecting the same type of anxiety (Ramos *et al.,* 2008). In fact, Acevedo *et al.* (2014) reported no correlation between the central time in the open field and the time spent in the bright side of the light/dark box, indicating that the type of anxiety assessed in these two paradigms must not be the same. If this were the case for the ethological-driven tests, it is also likely to envisage discrepancies

with other types of anxiety paradigms (i.e. novelty suppressed feeding and marble burying test).

A similar performance of WT and KO mice was detected in the novelty suppressed feeding, which is a test that allows a reliable assessment of mice´s performance under a conflictive-aversive context, and a good tool to measure hyponeophagia. Therefore, the anxiety assessed in the novelty suppressed feeding seems to be different to that evaluated in ethological tests (Calhoon and Tye, 2015).

5-HT$_4$ KO mice buried a lower number of marbles than their WT counterparts, a behavioural outcome that is in good agreement with our pharmacological studies: the acute administration of RS67,333 increased the burying behaviour in WT mice but not KO counterparts (data from our lab). A reduced marble burying behaviour is interpreted as an anxiolytic outcome, especially when testing drugs effects (i.e. after acute administration of fluoxetine or diazepam). Thus, outcome in the marble burying test appears to be reflecting an opposite response to that encountered in the open field. However, and as stated above, the outcome in the open field and light/dark box tests do not necessarily have to be the same than the observed in the marble burying test (Thomas *et al.,* 2009). In addition, it is worth to mention that the marble burying test is postulated to measure obsessive/compulsive behaviours (Londei *et al.,* 1998). This last assumption opens an interesting area of research on the role of the 5-HT$_4$ receptor in the pathophysiology and treatment of obsessive-compulsive disorders (OCDs), a topic yet to be explored. Whatever the case may be, our behavioural and pharmacological studies demonstrate that the marble burying behaviour is dependent on the presence of 5-HT$_4$ receptors.

Regarding the influence of 5-HT$_4$ receptors on the **depressive-related behaviours**, one of the most interesting finding is the anhedonia-like behaviour of KO mice as evidenced by a lower sucrose consumption (see summarizing figure 46). Moreover, they also showed an impaired nesting performance, which may reflect an "apathetic" or "self-neglect" behaviour (Filali *et al.,* 2009). Both findings reinforce the hypothesis that 5-HT$_4$ receptors could modulate some reward-related behaviours, and may suggest a pro-depressive-like trait of KO mice. High density of 5-HT$_4$ receptors are detected in the shell of nucleus accumbens of rodents (Compan

et al., 1996; Jean *et al.,* 2007), an area that control reward processes and, that it seems also to be involved in the regulation of feeding behaviour (Jean *et al.,* 2007; Jean *et al.,* 2012). We did not find changes in food consumption in the post-novelty suppressed feeding nor in the home-cage in KO mice. Therefore, we can conclude that the performance of KO mice in the sucrose and nesting tests is not reflecting an altered motivation for food feeding. In addition, we cannot rule out the participation of other brain areas in which 5-HT_4 receptors are also present. The nesting building behaviour is a highly complex feature that requires of an intact executive function (Jirkof *et al.,* 2014) and the hippocampal integrity (Deacon *et al.,* 2002). Hippocampal 5-HT_4 receptors play a unique and highly specialized role in synaptic information storage and cognition, and recently, the "pro-cognitive" effects of the 5-HT_4 receptor agonism upon the hippocampus-dependent learning tasks have been reviewed (Hagena and Manahan-Vaughan, 2017). A negative correlation between the 5-HT_4 receptor mRNA in the hippocampus and the anhedonia-like behaviour in rats has been reported in a maternal deprivation model (Bai *et al.,* 2014). Thus, a dysfunctional cognition circuitry in the hippocampus, due to the deletion of 5-HT_4 receptor, might also have influenced the performance of KO mice in the nesting paradigm.

A similar "behavioural despair" (forced swimming test) was observed in WT and KO mice, in good concordance with Kobayashi´s study (2011), in which a different strain of mice (C57BL/6) was used. However, it appears to be a non-expected finding when compared with the KO mice´s performance in the anhedonia-related paradigms. Besides, pharmacological studies reported a reduced immobility in the forced swimming test after acute administration of partial 5-HT_4 agonists in rats (Lucas *et al.,* 2007; Tamburella *et al.,* 2009). This discrepancy may be due to: a) methodological differences (including animal species and/or protocols); b) changes in the serotonergic system (Conductier *et al.,* 2006); and c) adaptive changes in brain neuroplasticity (as the one we further describe and discuss). Regarding the latter assumption, we postulate that these changes may install gradually over development in the 5-HT_4 constitutive KO mice and are sufficient to overcome some (i.e. "behavioural despair"), but not others (i.e. "anhedonia"), anxious-depressive manifestations.

Altogether, our findings and others´ reinforce the idea that distinct brain areas may be differentially engaged in each particular anxiety/depression related test. It is quite important to keep this in mind since different aspects of emotionality are covered by the umbrella term "anxiety" and “depression” in humans (File *et al.,* 1992). Moreover, it is also important to bring the expected contribution to the pre-clinical study of anxiety and depression given the multidimensional nature of emotional behaviours (Ramos, 2008).

5-HT$_4$ KO mice exhibit similar manifestations in anxiety/depression models

The above finding appears to be pointing to a certain emotional vulnerability of 5-HT$_4$ KO mice that could be more apparent when subjected to “pathological” circumstances. Therefore, WT and KO mice were subjected to two different models of chronic depression/anxiety: the chronic administration of corticosterone and the bilateral olfactory bulbectomy. From now on, we will refer to them as “the corticosterone model” and “the OBX model” (see summarizing figures 47 and 48).

When subjected to the **corticosterone model**, both genotypes exhibited a similar anxious/depressive phenotype in all the behavioural paradigms. This finding suggests that the chronic exposure to corticosterone appears to be affecting the hypothalamus-pituitary-adrenal axis in a similar way in both type of genotypes, and, therefore this would explain the similar susceptibility to the corticosterone-induced anxiety/depressive features. Some previous findings in the literature support this hypothesis. Corticosterone levels in 5-HT$_4$ KO mice are similar to those detected in WT mice in basal conditions and, even, when they are exposed to stressful events (Compan *et al.,* 2004).

In using the **OBX model**, we also circumvented how the 5-HT$_4$ receptors could be potentially involved in some traits of depression- and anxiety-like behavior. As already mentioned in the introduction, the olfactory bulbectomy induces a depressive/anxious-like phenotype that resembles the manifestations of depression in humans (Linge *et al*., 2013; Song and Leonard, 2005). Moreover, bulbectomized mice showed an increased expression of 5-HT$_4$ receptors in the hippocampus (Licht *et al.,* 2010a). Our study demonstrates that the constitutive absence of 5-HT$_4$ receptors did not modify the OBX-induced syndrome. In fact, bulbectomized 5-HT$_4$

KO mice presented a similar behavioural features than WT counterparts (i.e. hyperactivity, anxiety and anhedonia), thus, demonstrating the same susceptibility to the development and manifestations associated to this animal model of depression.

Comparison of the behavioural effects of RS67,333 and fluoxetine

This is the first time that the effects of chronic RS67,333 and fluoxetine are evaluated in chronic models of depression/anxiety and using 5-HT$_4$ receptor KO mice (see summarizing figures 47 and 48).

Chronic treatment with **RS67,333** induced an **anxiolytic**-like effect in **corticosterone-treated mice WT**, but not KO mice, as evidenced by an increased central activity in the open field; an effect already observed one week after the initiation of treatment. Therefore, we are the first demonstrating the 5-HT$_4$ receptor-dependent anxiolytic effect of the RS67,333 in a chronic model of depression/anxiety. An anxiolytic effect of RS67,333 in the open field and elevated plus maze after 7 days of treatment was also reported in the same model but using non-genetically modified C57BL/6 mice (Mendez-David *et al*., 2014). We confirm this 5-HT$_4$-receptor dependent **anxiolytic effect in the OBX-model**, though a later time-point of treatment (28 days). Our findings are in good agreement with studies demonstrating that other behavioral effects of RS67,333 seems to be dependent on the presence of 5-HT$_4$ receptors (Freret *et al*., 2012; Lamirault and Simon, 2001; Warner-Schmidt *et al*., 2009). However, as discussed below, we have also found some depression-related behavioural effects of the RS67,333 in that are not mediated by the 5-HT$_4$ receptor activation.

Chronic treatment with **fluoxetine** induced an "unexpected" **anxiogenic** effect (reduced central activity in the open field) in **naïve mice** of both genotypes. An anxiogenic effect of fluoxetine in naïve mice was also reported, not only in the open field, but also in the elevated plus maze (Baek *et al*., 2015). A paradoxical anxiogenic response of juvenile mice to fluoxetine was also reported, independently of the strains and tests used (Oh *et al.,* 2009). A higher, and significant, **anxiogenic effect** was also encountered in **corticosterone-treated KO** mice after 14 days of treatment with fluoxetine (as evidenced by a decreased central activity in the open

field). We speculate that it could be due to the innate anxiety that these animals already show, which could contribute to this outcome when they have been exposed to chronic corticosterone. This outcome might deserve further investigation since the initiation of treatment with antidepressant drugs can, in some patients, trigger or enhance anxious- and panic-like responses (Amsterdam *et al.,* 1994; Catalano *et al.,* 2000). In addition, in agoraphobic human patients, a type of anxiety related with thigmotaxis in the open field (Walz *et al*., 2016), fluoxetine only shows an anxiolytic effect at low doses (Perna *et al*., 2011), and perhaps the higher dose of fluoxetine used in our study could be detrimental to observe an anxiolytic effect in the open field. In contrast to our finding, an anxiolytic effect of fluoxetine was reported in corticosterone-treated mice (David *et al*., 2009; Mendez-David *et al.,* 2014). This discrepancy may be due to the different duration of the treatment (4 weeks in their study and 2 weeks in ours) and/or different mouse strains (C57BL/6 in their study and sv129 in ours). In **bulbectomized WT and KO mice**, chronic fluoxetine did not modify any of the central activity parameters of the open field.

Chronic treatment with **RS67,333** was not able to reverse the corticosterone-enhanced **anxiety** evaluated in the **novelty suppressed feeding test;** therefore, it showed lack of effect in this predictive paradigm of antidepressant efficacy. We can discard the anorexigenic effect of RS67,333 (Jean *et al*., 2007), which could have influenced negatively on the latency to feeding, since no changes in food consumption were observed in the post-NSF test. Anyway, we cannot rule out that a longer period of treatment with RS67,333, as the one reported by Mendez-David *et al.* (2014), were required to induce an anxiolytic/antidepressant effect in the NSF. Regarding the effects of **fluoxetine** treatment, we are providing a novel result regarding the onset of the effects of this SSRI in this paradigm known to assess the antidepressant efficacy of drugs. Indeed, we demonstrate that fluoxetine already produced a reversion of the corticosterone-enhanced anxiety at 7 days of treatment. This is "breaking" the assumption that, at least, 28 days of treatment with SSRIs are needed to observe an antidepressant effect when carrying out animal studies (Dulawa *et al*., 2004; Mendez-David *et al*., 2014; Santarelli *et al*., 2003). Indeed, this latter pattern was thought to resemble the time lag between initiating monoaminergic antidepressant therapy and the onset of therapeutic effects in

depressed subjects (Fitzgerald, 2014). However, this dogma has been questioned since the results from some meta-analysis reported an early antidepressant effect of the SSRIs (Posternak and Zimmerman, 2005; Taylor *et al*., 2006). We are also questioning it given the results obtained in the NSF with our mice; anyway, additional behavioural tests are needed to further characterized the onset of action of SSRIs.

Other interesting finding is the similar anxiolytic/antidepressant efficacy of **fluoxetine** in **corticosterone-treated 5-HT$_4$ KO and WT mice** as assessed in the NSF; this finding demonstrates that the effect of fluoxetine, at least in this behavioural paradigm, does not require the presence of 5-HT$_4$ receptors. This result appears to be in disagreement with that reported in the study of Mendez-David (2014). They demonstrated that the selective 5-HT$_4$ receptor antagonist GR125487 reversed the anxiolytic effect of fluoxetine, confirming the 5-HT$_4$ receptor-dependency. It is worth to mention that a pharmacological antagonism must not parallel the genetic deletion. For instance, it is possible that the affinity of GR125487 for 5-HT$_3$ receptors (Schiavi *et al*., 1994) could be behind this discrepancy since 5-HT$_3$ antagonism resulted in an anxiolytic effect in mice (Zhang *et al.,* 2001). Other hypotheses could be drawn, for example, the effect of fluoxetine in our KO mice could be due to monoaminergic adaptive mechanisms that might compensate the need of activating 5-HT$_4$ receptors for the antidepressant/anxiolytic effects of the SSRI.

In good agreement with the above NSF read-out, **RS67,333** administered chronically did not reverse the **OBX**-induced hyperactivity whereas **fluoxetine** fully attenuated it in a time-dependent fashion only in WT mice. Lucas *et al*. (2007) reported that 3-day treatment with RS67,333 reversed the hyperactivity of bulbectomized rats, claiming the faster antidepressant activity of this drug. The different specie used (rat in Lucas´ study and mice in ours) could account for this discrepancy. Indeed, several specie-specific differences regarding neurochemical and molecular changes, and the response to drugs have been reported (see review by Hendriksen *et al*., 2015). In addition, a strain-dependent effect must be considered (Hendriksen *et al*., 2015; Lucki *et al*., 2001); data from our group demonstrate that

7-day RS67,333 treatment resulted in the reversion of OBX-induced locomotor hyperactivity, though in C57BL/6 mice (Vidal *et al.,* 2013).

Regarding our results in KO mice in both animal models, it is quite intriguing the model-dependent effect of fluoxetine. The antidepressant was not effective in attenuating the hyperactivity of bulbectomized 5-HT_4 KO mice but it did reverse the enhanced anxiety of corticosterone-treated KO mice. A possible explanation may be related with the regulatory role of 5-HT_4 upon motor responses, which may contribute to the antidepressant effect induced by fluoxetine in the OBX model. In fact, high density of these receptors is reported in areas including the subtantia nigra (Waeber *et al*., 1994), where they modulate dopamine neurotransmission (Thorré *et al*., 1998), and therefore the locomotor behaviour in OBX mice.

Our findings showed that chronic RS67,333 does not exhibit antidepressant effect in the corticosterone- and OBX-models. We wondered whether the 5-HT_4 partial agonist would be able or not to modify other emotional features; therefore, in a separate set of animals, we evaluated the effect of RS67,333 in the sucrose intake test and splash test.

Chronic treatment with **RS67,333** did not reduce the corticosterone–induced **anhedonia** in WT mice as evidenced in the **sucrose intake test.** The lack of an anti-anhedonic effect of the 5-HT_4 partial agonist was confirmed in the bulbectomy model. These results are in disagreement with previous findings. One week of RS67,333 treatment reversed the anhedonia induced by the chronic unpredictable mild stress (Lucas *et al*., 2007) and the corticosterone (Pascual-Brazo *et al*., 2012) rat models. Data from our group also demonstrate that 7-day RS67,333 treatment resulted in the reversion of OBX-induced anhedonia, though in C57BL/6 mice (Vidal *et al.,* 2013). The discrepancy of our results with those in the literature could be due to different factors. Among them, the specie (rat in Lucas´and Pascual-Brazo´s studies and mice in ours), the mouse strain (C57BL/6 in Vidal´s study and sv129 in ours), and /or the doses used (i.e. 0.75 mg/kg/day in Lucas´ work *vs* 1.5 mg/kg/day in our study). An unexpected finding was the **"**anti-anhedonic" effect of **RS67,333** in **corticosterone-treated KO mice**. RS67,333 shows affinity for sigma-1 subtype receptors (Eglen *et al*., 1995a), whose activation may induce

antidepressant effects (Fishback *et al.,* 2010). Thus, it could be speculated that in the absence of 5-HT$_4$ receptors, some of the effects following the activation of sigma-1 subtype receptors by RS67,333 may be unveiled or become more apparent; an interesting pharmacological issue that deserve further investigation due to the research interest in developing new antidepressants by targeting sigma-1 receptor-mediated signalling (Fishback *et al.,* 2010). Chronic **RS67,333** also counteracted the corticosterone-induction decrease in the time of grooming in both genotypes as assessed in the **splash test.** This is a behavioural paradigm used to evaluate a mouse behaviour that it mimics the "apathy/self-neglect" of depressed patients (Diaz *et al*., 2016; Isingrini *et al*., 2010). The effect of RS67,333 upon the grooming behaviour has been already reported in C57BL/6 mice treated chronically with corticosterone and administered RS67,333 for 7 days (Mendez-David *et al*., 2014). In our study, the antidepressant effect of RS67,333 in the splash test was observed in both corticosterone-treated WT- and 5-HT$_4$-KO mice. As we mentioned above, and up to date, all the studies published state that the behavioral effects of RS67,333 are 5-HT$_4$-receptor dependent (Freret *et al*., 2012; Lamirault and Simon, 2001 and Warner-Schmidt *et al*., 2009). Again the complex receptor affinity profile of RS67,333 (i.e. 5-HT$_4$ partial agonist and affinity for sigma-1 receptors) may explain these findings. To the best of our knowledge, this the first study that shows the non 5-HT$_4$ receptor-dependent anti-anhedonic effects of the RS67,333.

2. REGULATION OF 5-HT$_{1A}$ RECEPTORS AFTER 5-HT$_4$ RECEPTOR DELETION

Changes in the 5-HT$_{1A}$ receptor expression and functionality have been linked to depression, anxiety and antidepressant effects (Savitz *et al.,* 2009). Given the anatomical and functional interaction between the different subtypes of serotonergic receptors, we anticipated that the deletion of 5-HT$_4$ receptors could alter the functionality of 5-HT$_{1A}$ receptors. In this sense, there are previous studies reporting that 5-HT$_4$ receptor KO mice presented alterations in different elements of the serotonergic neurotransmission (Conductier *et al.,* 2006). In this thesis, we aimed to extend this knowledge by evaluating the functionality of 5-HT$_{1A}$ receptors in 5-HT$_4$

receptor KO mice subjected to animal models of depression and treated with RS67,333 or fluoxetine (see summarizing figures 46, 47 and 48).

Adaptive changes in 5-HT$_{1A}$ functionality associated with 5-HT$_4$ receptors deletion

In order to assess the effect of the deletion of the 5-HT$_4$ receptor in naïve mice, we first performed *in vitro* (stimulation of [^{35}S]GTPγS binding by 8-OHDPAT) and *in vivo* (8-OH-DPAT-induced hypothermia) studies.

5-HT$_4$ KO mice showed a decrease in 8-OH-DPAT-induced stimulation of [^{35}S]GTPγS binding in the **dorsal raphe nucleus** (5-HT$_{1A}$ autoreceptors) without changes in the 5-HT$_{1A}$ receptor functionality in cortico-limbic projection areas. *In vivo* studies showed a similar 5-HT$_{1A}$ receptor-mediated hypothermic effect in both KO and WT naïve mice (no change in the 5-HT$_{1A}$ receptor functionality). This lack of concordance between the *in vitro* and *in vivo* results is quite surprising since 8-OH-DPAT-induced hypothermia in mice is supposed to be mediated by the activation of 5-HT$_{1A}$ autoreceptors (Martin *et al.,* 1992; Richarson-Jones *et al*., 2010). Our [^{35}S]GTPγS binding findings are in good agreement with the down-regulation (reduced protein expression) of 5-HT$_{1A}$ autoreceptor reported in these KO mice (Conductier *et al.,* 2006). In contrast to our results, the existence of a 5-HT$_{1A}$ autoreceptor hypersensitivity was demonstrated in KO mice, evidenced by a higher inhibition of the 5-HT neuronal firing after citalopram administration (Conductier *et al.,* 2006). Although it deserves further investigation, we postulate that the in vitro studies are assessing only the level of G-protein activation (especially Gi/o subunits) whereas in the in vivo studies the participation of other down-stream effectors (i.e. GIRK and N/P/Q type Ca^{2+} channels) cannot be discarded (Maejima *et al.,* 2013).

In the autoradiographic studies, we detected an increased basal [^{35}S]GTPγS binding in some brain areas of KO mice compared to WT littermates. It is assumed that an increase in the basal [^{35}S]GTPγS binding could be due to a higher constitutive activity of any type of G-protein coupled receptors, including the 5-HT$_{1A}$ receptor. Though it deserves further investigation, it could be speculated that this may be one

of the multiple compensatory mechanisms to overcome the lifelong loss of the 5-HT_4 receptor.

Regarding the behavioural implications, this reduced 5-HT_{1A} autoreceptor functionality in the KO mice may account, at least in part, for their enhanced innate anxiety (under a highly illuminated open field); in fact, genetic deletion of 5-HT_{1A} autoreceptors during the early developmental stage induces an anxious phenotype in the adulthood (Richardson-Jones *et al.,* 2011). Our 5-HT_4 KO mice also exhibited innate anhedonia (i.e. decreased sucrose intake) but not behavioural despair (forced swimming test), though the both manifestations are indicating a depression-like behaviour. Preclinical and clinical studies assessing the relationship between the 5-HT_{1A} autoreceptor expression/functionality and mood disorders draw inconclusive findings (Savitz *et al.,* 2009). We can only hypothesize, as in the case of the anxiety outcomes, that a developmental down-regulation of 5-HT_{1A} autoreceptors may be sufficient to ameliorate some dimensions of the umbrella term "depression" but not others (i.e. behavioural despair *versus* reward-related anhedonia). Moreover, the "innate" 5-HT_{1A} autoreceptor desensitization of KO mice could be interpreted as a compensatory mechanism with "anti-depressive properties", paralleling the 5-HT_{1A} autoreceptor desensitization observed after the chronic administration of antidepressants. Indeed, several studies in the literature (Blier and De Montigny, 1994; Castro *et al.,* 2003a; El Mansari *et al.,* 2005), and our pharmacological studies with fluoxetine (as discussed later) demonstrate a desensitization of 5-HT_{1A} autoreceptors associated with the antidepressant actions. Although it deserves further investigation, these changes on 5-HT_{1A} receptors in the dorsal raphe nucleus may represent an adaptive response to counterbalance the absence of the positive 5-HT_4 feedback on the firing activity of serotonergic neurons.

5-HT_{1A} receptors functionality in animal models of depression

In the two animal models of depression that we have used in this thesis, we found minor changes in 5-HT_{1A} receptor functionality, in some cases genotype-dependent and usually restricted to some brain areas.

A **5-HT_{1A} autoreceptor** desensitization was observed in **corticosterone-treated** WT mice, a finding that is in good agreement with previous studies using the same

corticosterone model (Hensler *et al*., 2007; Rainer *et al*., 2012). This desensitization of the 5-HT$_{1A}$ autoreceptors was also reported in other stress-related paradigms, such as the CUMS (Bambico *et al*., 2009), maternal deprivation (Leventopoulos *et al*., 2009) and social defeat (Kieran *et al*., 2010) rodent models. Human studies also demonstrate decreased expression of 5-HT$_{1A}$ autoreceptors in the midbrain of suicide victims with major depression (Boldrini *et al*., 2008), as well as in the dorsal raphe using PET studies (Drevets *et al*., 2000; Drevets *et al*., 2007; Meltzer *et al*., 2004); however, opposite changes have been reported (Parsey *et al.,* 2006; Stockmeier *et al.,* 1998). All these preclinical and clinical evidences suggest an association between the attenuation of 5-HT$_{1A}$ receptor function in serotonergic cell body areas and the manifestations of the depressive state. Chronic **corticosterone** did not alter the functionality of **DRN 5-HT$_{1A}$** receptors in **KO mice**, probably due to a "bottom effect" since they already exhibited an "innate" receptor desensitization; anyway, this differential outcome did not modify their corticosterone-induced syndrome since the depressive/anxiety manifestations were similar to that observed in corticosterone-treated WT mice. However, corticosterone-treated KO mice exhibited a higher susceptibility to the anxiogenic effect of chronic fluoxetine; this is an interesting issue since as stated above, some antidepressants could induce anxious- and panic-like responses at the beginning of the treatment (Amsterdam *et al.,* 1994; Catalano *et al.,* 2000).

The **olfactory bulbectomy** did not alter the functionality of **DRN 5-HT$_{1A}$ receptors** in WT mice. Previous studies in the OBX model reported decreased protein levels of the 5-HT$_{1A}$ receptor (Shin *et al*., 2017); however, a cell surface expression of 5-HT$_{1A}$ autoreceptors after olfactory bulbectomy was recently reported, indicating an increase in the protein levels (Riad *et al*., 2017). An autoradiographic study (as the one performed by us) demonstrated a desensitization of DRN 5-HT$_{1A}$ autoreceptors in bulbectomized mice (Linge *et al*., 2016). However, a different mouse strain (C57BL/6) was used in this study; in addition, mice were single housed, and it was reported that 5-HT$_{1A}$ receptor levels are modified after social isolation (Schiller *et al*., 2006). In **KO mice**, the **olfactory bulbectomy** increased the functionality of **5-HT$_{1A}$ autoreceptors** (+75% vs sham KO mice); indeed, the values of 8-OH-DPAT-stimulated [^{35}S]GTPγS binding in

bulbectomized KO mice were similar to those detected in bulbectomized WT mice. However, it must be noted that KO mice already exhibited a decreased functionality of 5-HT_{1A} autoreceptors before bulbectomy. We have not a clear explanation for the differential outcome in KO mice and WT counterparts after OBX surgery. However, it was reported that 5-HT_4 receptor binding is increased in the ventral hippocampus but decreased in the olfactory tubercles following olfactory bulbectomy in mice (Licht *et al.,* 2010a), two areas that may modulate, directly or indirectly, the activity of midbrain neurons. In addition, a differential regulation of other 5-HT receptor subtypes in KO mice might be possible. OBX surgery causes supersensitivity of cortical 5-HT_{2A} receptors derived from degeneration of neurons projecting from the olfactory bulb (Nakagawasai *et al.,* 2003), and it is well-known the role of these cortical 5-HT_{2A} receptors on the activity of DRN 5-HT neurons (Vázquez-Borsetti *et al.,* 2009). Therefore, we hypothesize that the lack of brain 5-HT_4 receptors in KO mice may have altered the functional interactions between the olfactory tubercles, forebrain and, especially those midbrain nuclei expressing 5-HT_{1A} autoreceptors.

In the case of 5-HT_{1A} **postsynaptic** receptors, both models induced changes that were restricted to some brain regions. Chronic **corticosterone** did not modify 5-HT_{1A} heteroreceptor functionality in WT mice, in concordance with the findings in a previous study (Hensler *et al*., 2007). However, it induced a hyperfunctionality of the 5-HT_{1A} receptors in the frontal cortex of corticosterone-treated KO mice; though the causes remain to be elucidated, the adaptive changes in forebrain areas, due to the absence of the excitatory role of cortical 5-HT_4 receptors, may be behind. Following **olfactory bulbectomy**, a hyperfunctionality of the 5-HT_{1A} receptor was observed in the CA3 hippocampal field in both genotypes. In the literature, the studies are inconclusive since no change (Gurevich *et al*., 1993) and reduced (Linge *et al*., 2016) functionality of hippocampal 5-HT_{1A} receptors have been reported in bulbectomized mice. In our case, we postulate that the 5-HT_{1A} hyperfunctionality could be a compensatory mechanism to the reduced levels of 5-HT found in the hippocampus of bulbectomized rats (Van der Stelt *et al*., 2005).

Taken together, these results demonstrate not only a model- but also a genotype-dependent regulation of 5-HT_{1A} receptors functionality in mice subjected to animal

models of depression. Anyway, the differences between WT and KO mice had not behavioural consequences since both genotypes manifested a similar anxious/depressive phenotype when they were exposed to corticosterone or subjected to the olfactory bulbectomy.

5-HT$_{1A}$ receptors functionality: effect of RS67,333 and fluoxetine

RS67,333 and fluoxetine differentially regulated the functionality of somatodendritic and cortico-hippocampal 5-HT$_{1A}$ receptors in both animal models.

5-HT$_{1A}$ autoreceptors. Chronic **RS67,333** reversed the **corticosterone**-induced desensitization of the somatodendritic DRN 5-HT$_{1A}$ receptors in WT mice. This is the first study demonstrating the effect of chronic RS67,333 upon the 5-HT$_{1A}$ receptor functionality in a depression/anxiety-like mouse model in which the response of the HPA axis is blunted. From a behavioural point of view, the reversal of the desensitization of 5-HT$_{1A}$ receptors induced by RS67,333 could be beneficial or detrimental depending on the type of behaviour. On the one hand, there are studies showing that signalling through endogenous 5-HT$_{1A}$ autoreceptors is necessary and sufficient for the establishment of normal anxiety-like behaviour (Richardson-Jones *et al.,* 2011); therefore, this "recovery" of the functionality of 5-HT$_{1A}$ receptors could explain the 5-HT$_4$ receptor-dependent anxiolytic of RS67,333 observed in corticosterone-treated mice. On the other hand, it could be detrimental to produce an antidepressant effect in the novelty suppressed feeding test since it was reported that the higher expression and functionality of 5-HT$_{1A}$ autoreceptors in mice resulted in poor antidepressant response (Garcia-Garcia *et al*., 2016b; Richardson-Jones *et al*., 2010). Chronic **fluoxetine** treatment significantly enhanced the **corticosterone-**induced 5-HT$_{1A}$ autoreceptor desensitization in WT mice, in good agreement with the desensitization reported in *in vivo* 8-OH-DPAT-induced hypothermia assays (Rainer *et al.,* 2012). Finally, in **corticosterone-treated KO mice**, the chronic administration of **RS67,333 or fluoxetine** did not alter 5-HT$_{1A}$ autoreceptors functionality; this outcome indicates that the adaptive changes induced by chronic corticosterone upon the DRN 5-HT$_{1A}$ receptors require the presence of brain 5-HT$_4$ receptors.

Chronic treatment with **RS67,333** did not have any impact upon the 5-HT_{1A} autoreceptor functionality in **bulbectomized mice.** In contrast, chronic treatment with **fluoxetine** induced a desensitization of 5-HT_{1A} autoreceptors in bulbectomized mice of both genotypes (non 5-HT_4-receptor dependent effect). This fluoxetine-induced desensitization is in good concordance with the findings reported in a previous study using bulbectomized rats and published by our group (Rodríguez-Gaztelumendi, 2010).

Regarding the effects of **RS67,333 and fluoxetine in naïve mice**, the findings did not parallel those described in corticosterone-treated mice. In the one hand, chronic RS67,333 treatment was associated with a 5-HT_4-receptor dependent desensitization of 5-HT_{1A} autoreceptors, in line with a previous study in naïve rats (Lucas *et al.,* 2007). In the other hand, chronic fluoxetine (2 weeks) did not alter the 5-HT_{1A} autoreceptor functionality in naïve WT and KO mice. However, a reduction in the capacity of the 5-HT_{1A} autoreceptor to activate G protein was detected in rats after the same time of treatment (Castro *et al.,* 2003a; Hensler, 2002). Nevertheless, it is important to keep in mind that the 5-HT_{1A} autoreceptor desensitization is not always a required phenomenon to induce the antidepressant effects. For example, sertraline and venlafaxine, SSRIS antidepressants widely used to treat mood disorders, do not reduce the efficiency of G-protein coupling of these somatodendritic receptors (Rossi *et al*., 2006 and 2008). There is also an important methodological aspect: the studies that assess the level of receptor-G protein interaction (as in Rossi *et al*., 2006) do not always show the same results than those studies that evaluate the 5-HT_{1A} receptor-mediated DRN neuronal activity (as in Rosi *et al.,* 2008). In **naïve KO**, we were expecting to find a desensitization of 5-HT_{1A} autoreceptors following chronic fluoxetine treatment since it has been reported that these mice exhibit an increased serotonin transporter (5-HTT) density in the DRN (Conductier *et al.,* 2006). Thus, its blockade by fluoxetine could lead to increased synaptic levels of 5-HT and, subsequently, to an adaptive 5-HT_{1A} autoreceptor desensitization. However, Conductier *et al.* (2006) have reported that KO mice present low basal 5-HT levels, which could counterbalance the increased 5-HT levels induced by fluoxetine, explaining that 5-HT_{1A} autoreceptor

"normosensitivity"; microdialysis studies aimed to evaluate the effect of fluoxetine on DRN 5-HT levels in WT and KO mice are needed to confirm this hypothesis.

5-HT$_{1A}$ heteroreceptors. Chronic administration of RS67,333, in general terms, did not modify the functionality of postsynaptic 5-HT$_{1A}$ receptors in both animal models. Indeed, **RS67,333** only reversed, in a non 5-HT$_4$-dependent manner, the hypersensitivity of 5-HT$_{1A}$ receptors in the CA3 field observed in **bulbectomized** mice, though not associated with behavioural effects.

Chronic **fluoxetine** did no modify 5-HT$_{1A}$ heteroreceptors functionality in **bulbectomized** mice. However, its administration to **corticosterone-treated** mice was associated to a 5-HT$_4$ receptor-dependent desensitization of 5-HT$_{1A}$ heteroreceptors in many brain areas, especially in the frontal cortex and CA1 fields. This attenuated functionality of 5-HT$_{1A}$ heteroreceptors by the SSRI was confirmed in non-corticosterone-treated (naïve) WT and KO mice.

All these conflicting findings in our study are in parallel with the inconclusive literature. After long-term SSRI treatment, agonist-induced [^{35}S]-GTPγS binding data showed an increase (Castro *et al*., 2003a; Moulin-Sallanon *et al*., 2009; Shen *et al*., 2002) or no change (Hensler, 2002; Pejchal *et al*., 2002) in rodents and after chronic mild stress (Burke *et al*., 2013). In addition, human studies investigating the density of postsynaptic 5-HT$_{1A}$ receptors have not provided a clear evidence of their regulation by antidepressants. For example, in one study of SSRI treatment effects on human 5-HT$_{1AR}$ binding potential, no treatment-associated changes were evident in regions where the 5-HT$_{1A}$ receptor is expressed postsynaptically (Sargent *et al.*, 2000). In any case, postsynaptic 5- HT$_{1A}$ receptors have been shown to be unaltered or reduced in depressed patients, and this alteration is not sensitive to antidepressant treatment (Bhagwagar *et al.,* 2004). Our pharmacological studies are providing further information about the effects of antidepressant drugs on the 5-HT$_{1A}$ auto- and heteroreceptor functionality, adding more controversy to this topic.

3. CHANGES IN NEUROPLASTICITY

Adaptive changes in BDNF/trkB

As stated in the introduction section, there are many studies in the literature supporting the involvement of BDNF, and its cognate receptor trkB, in the pathophysiology of the depression and the mechanism of action of antidepressants (see reviews by Duman and Monteggia, 2006; Castrén and Kojima, 2017). In our study, using a genetic animal model, we are providing new findings about the role of 5-HT_4 receptors on the regulation of BDNF/TrkB signalling pathway in animal chronical exposed to corticosterone or subjected to olfactory bulbectomy, and following treatment with RS67,333 or fluoxetine (see summarizing figures 46, 47 and 48).

Naïve 5-HT_4 receptor KO mice. The absence of 5-HT_4 receptors altered brain BDNF/trkB signalling pathway in restricted areas, especially the hippocampus. These mice exhibited an increased BDNF mRNA expression in the dentate gyrus in the hippocampus accompanied with a reduction in trkB receptor mRNA expression in other hippocampal fields, the amygdala and the hypothalamus. According to this finding, it is tempting to hypothesize a relationship between the behavioural manifestations of 5-HT_4 KO mice and these molecular changes. The overexpression of BDNF in the hippocampus leads to an anxious-like phenotype (Casarotto *et al.,* 2012, Deltheil *et al*., 2008; Deltheil *et al.,* 2009), which is in line with the anxiety-like behaviour our KO mice exhibited. More difficult is to establish a correlation with the depression-related behaviour of 5-HT_4 KO mice. Increased hippocampal BDNF expression was associated with the anhedonia observed in the chronic unpredictable mild stress (Boulle *et al.,* 2014) and OBX (Hellweg *et al.,* 2007) models; in our case, whether this could represent a causal or compensatory relationship is an issue that remains to be elucidated. In the section of results on the effects of RS67,333 and fluoxetine we describe that KO-mice chronically treated with vehicle presented a similar BDNF mRNA expression in the dentate gyrus than vehicle WT- counterparts; this is in discrepancy with the increased BDNF levels detected in *naïve* KO mice in the same brain area. We postulate a higher susceptibility of KO mice to the handling associated with the chronic procedures

carried during drug treatment (i.e. saline injection, sham-operation, battery of behavioural tests…).

5-HT$_4$ receptors and animal models. Regarding the regulation of the levels of BDNF/trkB mRNA expression in animal models of depression, we provide experimental evidences of model-dependent changes, though restricted to some brain areas.

As far as we know, only one study (Jacobsen and Mørk, 2006), using the same technique, has already evaluated BDNF mRNA expression in corticosterone-treated animals, though in a different specie (rats). Chronic **corticosterone** treatment had no effect on **BDNF** mRNA in both genotypes. However, corticosterone-treated 5-HT$_4$ KO mice presented lower levels of BDNF than corticosterone-treated WT mice at the level of the medial prefrontal cortex, and in the CA1 field and dentate gyrus of the hippocampus. This, again, may suggest their higher susceptibility to stress-related events, as already stated. A small reduction in BDNF mRNA was reported in the CA3 of the hippocampus, but not in the dentate gyrus and frontal cortex, associated to a decreased BDNF protein levels in total hippocampus in rats (Jacobsen and Mørk, 2006). Other studies reported decreased levels of BDNF after chronic corticosterone administration but using different methodological approaches: the mRNA detection by quantitative PCR (Mao *et al*., 2014; Yi *et al*., 2012) and the determination of protein levels by ELISA/western blot (Demuyser *et al*., 2016; Mao *et al*., 2014; Yi *et al*., 2012). These results are not in line with our findings but we must take into account that our *in situ* hybridization assays are providing higher anatomical resolution than the other techniques in which samples homogenates were used. In addition, the levels of BDNF protein do not always parallel those of BDNF mRNA. Chronic corticosterone treatment did not alter the levels of expression of trkB mRNA in the two genotypes, a finding that confirms the data obtained in other studies and reporting the absence of **trkB** mRNA regulation; however, decreased trkB protein levels were also reported (Kutiyanawalla *et al*., 2011; Yi *et al*., 2012). Our findings point to a lack of regulation on BDNF/trkB signalling after chronic corticosterone administration, a depression/anxiety-like model in which the response of the HPA axis is blunted.

In **bulbectomized** mice**,** the only significant finding was the increased **BDNF** mRNA expression in the pyriform cortex in both genotypes, with no changes in the hippocampus. In line with our finding, increased BDNF protein levels, measured by immunoassay techniques, were reported in bulbectomized mice (Hellweg *et al*., 2007); however, decreased levels are also reported, but in bulbectomized rats (Hendriksen *et al*., 2012). This highlight the specie-dependent differences on the adaptive changes in the OBX model (Hendriksen *et al*., 2015). Olfactory bulbectomy may induce the differentiation of immature neurons on the pyriform cortex, a region that it is involved in the olfactory recognition process (Gómez-Climent *et al.,* 2011). Hence, an increase in the levels of BDNF in the pyriform cortex may favour this adaptive differentiation process, in view of the role of BDNF in the regulation of neuronal differentiation (Chen *et al*., 2013). Olfactory bulbectomy increased the levels of **trkB** mRNA in the nucleus accumbens in both genotypes, a molecular event that could be linked to some of the bulbectomy-induced depressive-like manifestations, especially the anhedonia. A single bilateral infusion of a trkB receptor antagonist into the nucleus accumbens exerted antidepressant-like effects in the learned helplessness rat model of depression (Shirayama *et al*., 2015) and in an inflammation-induced model of depression (Zhang *et al*., 2015). Thus, and conversely, we can postulate that the increased levels of trkB in the nucleus accumbens of bulbectomized mice could play a role in some of the depressive features of the OBX syndrome.

Effects of chronic treatment with RS67,333 and fluoxetine. Both drugs altered BDNF mRNA expression in both depression/anxiety models, and we demonstrate differential changes depending on the drug and the model studied.

RS67,333 reduced, in a non 5-HT_4 receptor-dependent fashion, the levels of BDNF mRNA in the CA1 and the dentate gyrus in **corticosterone**-treated mice. Conversely, **fluoxetine** largely increased BDNF mRNA levels in the dentate gyrus in the corticosterone-treated mice of both genotypes. This opposite regulation might explain, at least in part, the different behavioural profile of each drug: a) the antidepressant efficacy of fluoxetine, but not RS67,333 in the novelty suppressed feeding test; and b) their different effects upon the central activity (anxiety-related response) in the open field. It has been reported that BDNF mimics antidepressant-

like effects in several behavioural experimental paradigms (Murakami *et al.,* 2005; Grønli *et al.,* 2006), and the overexpression of BDNF in the hippocampus induces antidepressant effects (Deltheil *et al.,* 2009). Furthermore, knockdown of BDNF in the dentate gyrus in rats produces depression-like effects (Taliaz *et al.,* 2010). Nonetheless, there are animals studies describing opposite roles of BDNF in the VTA-NAc circuit compared with the hippocampal-prefrontal circuit (Eisch *et al.,* 2003). Decreased BDNF expression in dentate gyrus, but not in the CA1 hippocampal, was reported to induce resistance to the antidepressant treatments in mice (Adachi *et al.,* 2008). An overexpression of BDNF in the hippocampal astrocytes produced an antidepressant effect in the novelty suppressed feeding test, and a good correlation was found between the response in this test and the number of tertiary dendrites observed in this overexpressed mice (Quesseveur *et al*., 2013). In the corticosterone model, the effects of fluoxetine in the novelty suppressed feeding test are reported to be neurogenesis-dependent (David *et al*., 2009). All these evidences supports the hypothesis that this increased BDNF in the dentate gyrus, through a neurogenesis-dependent mechanism, could mediate the antidepressant effects of fluoxetine in this paradigm. In our study, a correlation analysis revealed a negative relationship between the latency to feeding and the BDNF mRNA expression in the dentate gyrus. This was particularly noticeable when the individual data from corticosterone-treated mice were plotted, demonstrating that high corticosterone-enhanced anxiety was correlated with low BDNF mRNA expression in this hippocampal area. This is even more clear-cut in those corticosterone-treated mice that received fluoxetine. These findings highlight the implication of this neurotrophic protein in the antidepressant outcome of the SSRI when this drug is assessed in the novelty suppressed feeding under pathological conditions. On the contrary, the reduction of BDNF induced by the chronic treatment with RS67,333 might explain the absence of efficacy of this drug in the novelty suppressed feeding. Thus, fluoxetine increased BNDF levels in corticosterone-treated mice, in association with its efficacy in the NSF test, as evidenced by a reversion of the enhanced anxiety induced by this model. It has been reported that the overexpression of BDNF in the hippocampus induces an anxiogenic-like behavior in the open field (Deltheil *et al*., 2008; Deltheil *et al*., 2009), and light/dark box (Casarotto *et al*., 2012), though an anxiolytic effect in the

elevated plus maze (Bahi, 2017). In our case, we observed an increase of BDNF in the dentate gyrus after fluoxetine treatment in both corticosterone-treated genotypes. This molecular change might be associated with the behavioural outcome of fluoxetine in the open field test (i.e. anxiogenic- rather than anxiolytic-like effects), especially in corticosterone-treated KO mice. On the contrary, chronic RS67,333 treatment induced a 5-HT_4-receptor dependent downregulation of hippocampal BDNF levels, which could be correlated with its anxiolytic effect observed in corticosterone-treated WT mice in the open field. These opposite effects of BDNF levels in anxiety and depression were reported previously (Govindarajan *et al*., 2006); in this study, the anxiogenic effect of BDNF was associated with an increase in BDNF levels in the amygdala. Unfortunately, we could not measure BDNF expression in this brain region due to the lack of enough signal to make comparisons between the different experimental groups. We postulate that, similarly to that observed in the hippocampus, chronic RS67,333 may downregulate BDNF levels in the amygdala, inducing an anxiolytic effect in the open field. Moreover, chronic fluoxetine was reported to increase BDNF mRNA levels in the amygdala (Balu, 2010; Mikics *et al*., 2017; Nowacka *et al*., 2014), a neuroplasticity change that could be harmful and that would explain its anxiogenic effect in the open field.

Interestingly, the fluoxetine-induced regulation of BDNF in *naïve* (non-CORT) mice did not seem to be relevant for their behavioural performance in the NSF. This emphasises that we must be careful when correlating the molecular actions of a given drug with its behavioural effects. Therefore, we should pay attention to the type of study (*naïve* or "healthy" animals or subjected to "pathological" conditions) before stating any translational implications. Other interesting issue is the increased BNDF expression induced by fluoxetine in KO mice. In fact, Imoto *et al*. (2015), using 5-HT_4 receptor KO mice, introduced a potential role of the 5-HT_4 receptors in fluoxetine-induced neurogenic activity and granule cell dematuration in the dentate gyrus. However, other neurothropic/neuroplastic proteins may be involved in the non 5-HT_4-receptor dependent pro-neurogenic effect of fluoxetine. In fact, we clearly demonstrate an increased BDNF not only in corticosterone-treated WT-but

also KO mice administered fluoxetine, a molecular change associated with its antidepressant effect.

RS67,333 and **fluoxetine** exhibited opposite changes on BDNF levels in **bulbectomized** mice: a) RS67,333 enhanced while fluoxetine reversed the OBX-induced BDNF upregulation detected in cortical areas (i.e. pyriform cortex); and b) RS67,333 increased while fluoxetine decreased BNDF levels in the hippocampus.

The RS67,333-induced increased levels of BDNF observed in bulbectomized mice were not associated with any beneficial behavioral outcome, which is in contrast to some postulates of the neurotrophic hypothesis. Other unexpected finding was the fluoxetine-induced reduction of hippocampal BDNF levels in bulbectomized mice associated with its clear-cut antidepressant effect (i.e. reversion of OBX-hyperactivity). Moreover, chronic fluoxetine reversed the OBX-induced increased BNDF levels detected in the pyriform cortex. These findings demonstrate that fluoxetine down-regulated the levels of this neurotrophin in the OBX-model. It is noteworthy to mention that, as nicely reviewed (Hendriksen *et al*., 2015), there are specie-dependent differences on the adaptive changes following olfactory bulbectomy. For instance, olfactory bulbectomy increased BDNF in cortical and hippocampal areas in mice but decreased it in rats (Hellweg *et al*., 2007; Hendriksen *et al*., 2015). Regulation of BDNF in bulbectomized mice has been proposed to be a mechanism to counteract the surgery damage and fluoxetine could have abolished the need for the adaptive response of neuroplasticity mechanisms that occur after surgery (Freitas *et al*., 2013). If we keep in mind this assumption, it is plausible to observe no change or even upregulated BDNF levels after RS67,333 treatment associated with its lack of antidepressant effect. Furthermore, our studies demonstrated a positive correlation between the levels of BDNF in the dentate gyrus and total distance travelled by bulbectomized mice in the open field. This reinforces the assumption that a reduction of hippocampal BDNF could be beneficial to reduce the locomotor hyperactivity of bulbectomized animals, as chronic fluoxetine did, in contrast to chronic RS67,333.

RS67,333 and fluoxetine modified the expression of **trkB** only in the nucleus accumbens, a brain region known to be strongly implicated in depression. **RS67,333**

treatment induced a downregulation of the levels of trkB in the nucleus accumbens in **corticosterone**-treated WT mice. In **bulbectomized** WT mice, **RS67,333 and fluoxetine** also reduced trkB levels in this nucleus. It has been shown that the infusion of a trkB antagonist in the nucleus accumbens induces an anxiolytic effect in the open field in chronic stressed rats (Azogu and Plamondon, 2017). Thus, RS67,333-induced downregulation of trkB in this nucleus might be correlated with its anxiolytic effect in the open field and its anti-anhedonic effect. The vast majority of trkB KO mice do not present a depressive or anxious phenotype (Lindholm and Castrén, 2014); though the conditional deletion of trkB from the newly-born neurons in the adult dentate gyrus increases anxiety-like behavior (Bergami *et al.,* 2008). Interestingly, the infusion of the trkB antagonist does induces not only an anxiolytic effect in stressed rats (Azogu and Plamondon, 2017) but also an antidepressant effect in some models of depression (Shirayama *et al*., 2015; Zhang *et al*., 2015). Then, we postulate that the reduction in trkB signalling induced by chronic fluoxetine in corticosterone-treated and bulbectomized mice could be linked to in its antidepressant effect on these animal models.

Taken all together, our findings provide new insights regarding the role of 5-HT_4 receptors upon the regulation of BDNF/TrkB signalling pathway on depression and, especially, on the mechanisms of action of classical and putative antidepressants drugs.

Adaptive changes in Arc

Activity-regulated-cytoskeleton associated protein **(*Arc*)** has an important role in the regulation of dendritic spine density and morphology; thus, it is postulated to be involved in the pathophysiology of depressive disorders and in the mechanism of action of antidepressant drugs (Li *et al.,* 2015).

Naïve 5-HT_4 receptor KO mice. 5-HT_4 receptor KO showed increased levels of this protein in the cingulate cortex, CA1 and CA3 hippocampal field. Although these changes may account for the anhedonia exhibited by these KO mice, it is not easy to explain the correlation between the behaviour and these molecular changes. 5-HT_4 KO mice are reported to exhibit an increased muscarinic neurotransmission (Segu *et al.,* 2010), which appeared to be responsible for the increased levels of *Arc* (and

BDNF); a direct relationship between cholinergic transmission and these neuroplasticity proteins has been reported in some behavioural studies in mice (Gil-Bea *et al.,* 2011). We postulate that the increased *Arc* mRNA expression due to the absence of 5-HT_4 receptors could mean a compensatory mechanism for the lifelong loss of 5-HT_4 receptors. Indeed, an enhanced expression of *Arc* mRNA in cortical and hippocampal areas has been described in rodent models of depression (Boulle *et al.,* 2014; Coppens *et al.,* 2011).

5-HT_4 receptors deletion and animal models. To the best of our knowledge, this is the first study analyzing *Arc* mRNA expression in these animal models. *Arc* levels were not modified in WT mice in either **corticosterone**-treated or **bulbectomized**. This lack of regulation is an unpredicted result in these animals showing a depressive/anxious phenotype, according to the literature in this field; however, it must be noted that the findings depend on the brain areas, animal models and species studied (reviewed in Li *et al.,* 2015). For instance, low levels of *Arc* mRNA were reported in the frontal cortex and the hippocampus following chronic social isolation stress in mice (Ieraci *et al*., 2016), but increased levels were found in young rats following social defeat (Coppens *et al*., 2011) and in mice subjected to chronic unpredictable mild stress (Boulle *et al.,* 2014). In our case, the absence of changes in *Arc* mRNA expression in these animal models may be correlated with the lack of relevant alterations in BDNF expression in both animal models, since *Arc* mRNA levels are regulated by BDNF signalling (Yin *et al*., 2002; Zheng *et al*., 2009). In KO mice; chronic exposition to corticosterone reduced *Arc* mRNA expression in some cortical areas, but not in the hippocampus. According to the above cited literature, this would imply a higher susceptibility to the corticosterone model, but this was not the case.

Effects of chronic treatment with RS67,333 and fluoxetine. **RS67,333** downregulated *Arc* mRNA expression only in CA3 hippocampal field in **corticosterone**-treated mice. Chronic **fluoxetine** reduced *Arc* mRNA expression it all across the brain, especially in the hippocampus, in corticosterone-treated mice. Little is known about the regulation of *Arc* by classical and putative antidepressants in this animal model of depression. Indeed, there is only one study showing that fluoxetine downregulates the expression of *Arc* in the lateral amygdala in the

corticosterone model (Monsey *et al*., 2014). Agomelatine, a non-SSRI antidepressant, reversed the upregulation of *Arc* mRNA expression induced by the CUMS model, a paradigm quite similar to our corticosterone model (Boulle *et al*., 2014). These findings in both studies are in concordance with ours. Other interesting outcome in our study is that the regulation of *Arc* hippocampal levels by fluoxetine in the corticosterone model did not require the presence of 5-HT_4 receptors, similarly to what happened in the case of BDNF. Therefore, the hippocampal neuroplasticity changes that depend on BDNF and *Arc* signalling and related to the behavioural effects of fluoxetine may still be present in the absence of 5-HT_4 receptors, an issue that deserves further investigation.

Rergarding the findings on the **OBX model**, this is the first study evaluating drug effects on *Arc* mRNA expression in this model. An opposite regulation is observed depending of the drug administered. **Fluoxetine** reduced *Arc* mRNA expression in all the cortical and hippocampal areas studied, a finding that is in concordance with our results in the corticosterone model and other´s studies (Boulle *et al*., 2014; Monsey *et al*., 2014) in the literature. In contrast, **RS67,333** increased *Arc* levels in the dentate gyrus in bulbectomized WT mice. We think that the RS67,333-induced upregulation of *Arc* levels may explain, together with its effect in BDNF levels, its lack of antidepressant effects in bulbectomized mice (i.e. inability to attenuate OBX-related hyperactivity).

We have also detected a differential regulation of *Arc* mRNA by fluoxetine in vehicle treated mice (upregulation) in comparison with animals exposed to corticosterone or subjected to bulbectomy (downregulation in both models). The upregulation observed in these "non-modelled" mice is consistent with that previously reported in *naïve* animals by other groups (Alme *et al*., 2007; De Foubert *et al*., 2004; Ferres-Coy *et al*., 2016). On the other hand, the fluoxetine-induced downregulation of *Arc* in our animal models must be seem as a beneficial molecular change since neuroplasticity also includes the elimination of synapsis that do not mediate valuable information (Castrén and Antila, 2017). In this context, a reduction of the expression of *Arc* could be beneficial to alter some harmful connections that could be generated in the depressive state, in order to modulate the aberrant functionality of some brain circuitries associated with the mood disorders

(Cao *et al*., 2014). Again this mismatch between "healthy" and "depressed" mice, and, as stated above, reinforce the idea of distinguishing between healthy and disease-modelled conditions when looking at the molecular changes associated to the behavioural antidepressant effects.

Adaptive changes in mTOR signalling

In the last years, mTOR signaling have gained relevance in depression and in the mechanism of action of the antidepressant drugs, especially after the discovery of the fast actions of ketamine through the modulation of the glutamatergic neurotransmission (Li *et al.,* 2010). The hippocampal 5-HT$_4$ receptors are mainly located in glutamatergic neurones (Peñas-Cazorla and Vilaró, 2014), and glutamatergic receptors are known to regulate mTOR signalling pathways (Page *et al.,* 2006). Moreover, a reduced mTOR signalling was found in the cortex frontal of depressive patient (Jerignan *et al.,* 2011) and in the hippocampus of mice subjected to chronic unpredictable mild stress (Liu *et al.,* 2015).

The behavioural findings in our *naïve* KO mice are in line with the above assumption since an **impaired mTOR signalling** was observed in 5-HT$_4$ KO mice associated with their "innate" anxiety and anhedonia. According to these results, we anticipated an impaired mTOR pathway in rodents exposed to animal models. Unexpectedly, no significant changes were detected in the values of pmTOR/mTOR ratio in the hippocamnpus of both genotypes after **chronic corticosterone or olfactory bulbectomy.** However, a decrease in the phosphorylation of mTOR in the hippocampus was reported in the corticosterone model (Pazini *et al*., 2016). An increase in mTOR signaling in the mPFCx in bulbectomized rats (Jiménez-Sánchez *et al*., 2016) and mice (Linge *et al.,* 2015) was also described. We think that there several methodological issues accounting for the discrepancy of our findings with the mentioned studies. First, the animal specie (mice *vs* rat in Jiménez-Sánchez´s work). Second, the mouse strain (Swiss in Pazini´s *vs* our sv129 mice). Third, the gender (female in Pazini´s *vs* male in our study), since sex differences in animal models of depression have been reported (Kokras and Dalla, 2014). Finally, the brain region analyzed (frontal cortex in Jiménez-Sánchez´s and Linge´s studies *vs*

hippocampus in our case), in line with the reported region dependent modulation of the functionality of mTOR (Liu *et al*., 2015).

Not so surprising was the lack of mTOR regulation by chronic **RS67,333** treatment in all the experimental groups (*naïve* and models). Thus, the absence of modulation of mTOR signaling by RS67,333 could be one of the molecular substrates explaining the weak antidepressant effects of this compound in our assays. In contrast, the antidepressant outcome of fluoxetine is in good agreement with its effect on mTOR signalling since chronic administration of the SSRI in mice attenuated the CUMS-induced mTOR phosphorylation reduction (Liu *et al.,* 2015). This latter finding is in line with the required activation of the mTOR pathway to observe antidepressant effects after deep brain stimulation (Jiménez-Sánchez *et al*., 2016) and ketamine administration (Li *et al*., 2010).

Adaptive changes in Wnt/β-catenin signalling

Wnt/β-catenin is an intracellular signalling pathway implicated in hippocampal proliferation (Zhang *et al.,* 2011) and the mechanism of action of antidepressants (Mostany *et al.,* 2008; Pilar-Cuellar *et al.,* 2014).

Our results demonstrate that *naïve* WT and 5-HT$_4$ receptor KO mice presented similar expression levels of β-catenin and its phosphorylated form, suggesting a "normal" functioning of this neuroplasticity-related pathway after genetic deletion of 5-HT$_4$ receptos. Moreover, *naïve* 5-HT$_4$ KO mice showed no changes in hippocampal proliferation, a cellular process in which mTOR and Wnt/β-catenin are implicated. This is an unexpected finding since there are some animal studies showing that 5-HT$_4$ receptors are critically involved in the proliferative/neurogenic effects of antidepressants (Imoto *et al.,* 2015; Mendez-David *et al.,* 2014). However, our results are in good concordance with those reported in a previous study in other strain of *naïve* 5-HT$_4$ KO mice (Imoto *et al.,* 2015). It cannot be ruled out that the increased BDNF levels in the dentate gyrus and/or a normo-functional Wnt/β-catenin signalling in the constitutive KO mouse may explain, at least in part, the lack of impaired hippocampal proliferation.

In the **corticosterone model,** we found increased β-catenin levels but a normal p-β-catenin/β-catenin ratio, indicating an increase in p-β-catenin levels. We propose that the finding of a "normal" ratio could be due to adaptive changes in GSK-3-β activity, since an increased GSK-3-β phosphorylation activity was reported in the hippocampus of corticosterone-treated mice (Chen *et al*., 2014). In **bulbectomized mice**, the reduction of β-catenin protein levels observed in the hippocampus is consistent with a previous study in bulbectomized rats (Rodríguez-Gaztelumendi *et al.,* 2010). Moreover, this reduction fits well with the increase in the ratio p-β-catenin/β-catenin observed, since this increase could promote its degradation (Ge *et al*., 2016).

Chronic treatment with **RS67,333** did not modify the levels of β-catenin and the p-β-catenin/β-catenin ratio in *naïve* mice, which is in line with a previous study in rats (Pascual-Brazo *et al*., 2012). Moreover, chronic RS67,333 had not effect on p-β-catenin/β-catenin ratio in corticosterone-treated and bulbectomized mice, suggesting that RS67,333 does not modify β-catenin signalling. It is known that the antidepressant effect of the citalopram (Liu *et al*., 2012) and ketamine (Beurel *et al*., 2011) requires of the inhibition of GSK3β and, the subsequent activation of β-catenin.

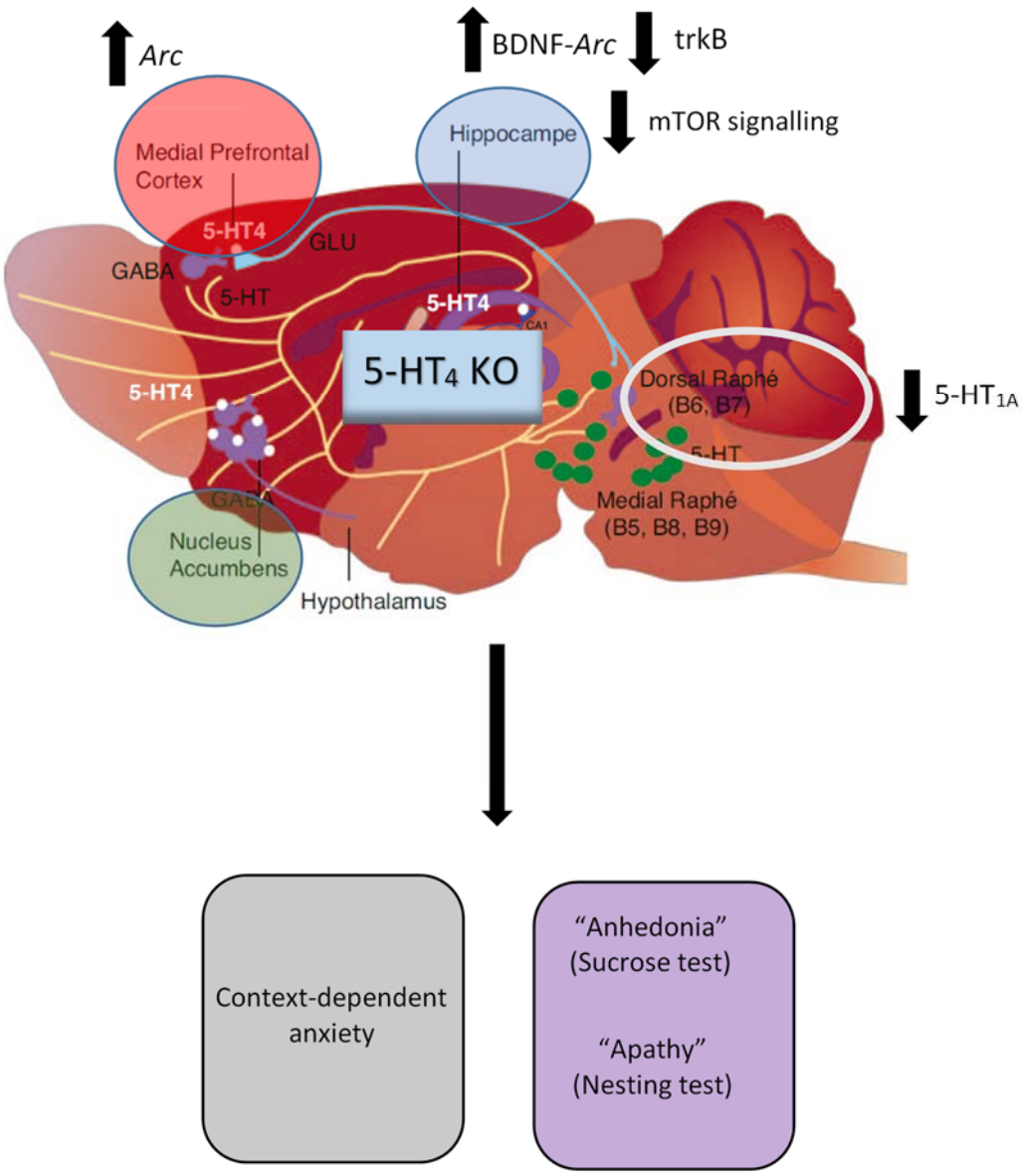


Figure 46. Scheme summarizing the neurochemical and molecular changes in 5-HT$_4$ receptor *knockout* mice associated with their behavioural phenotype.

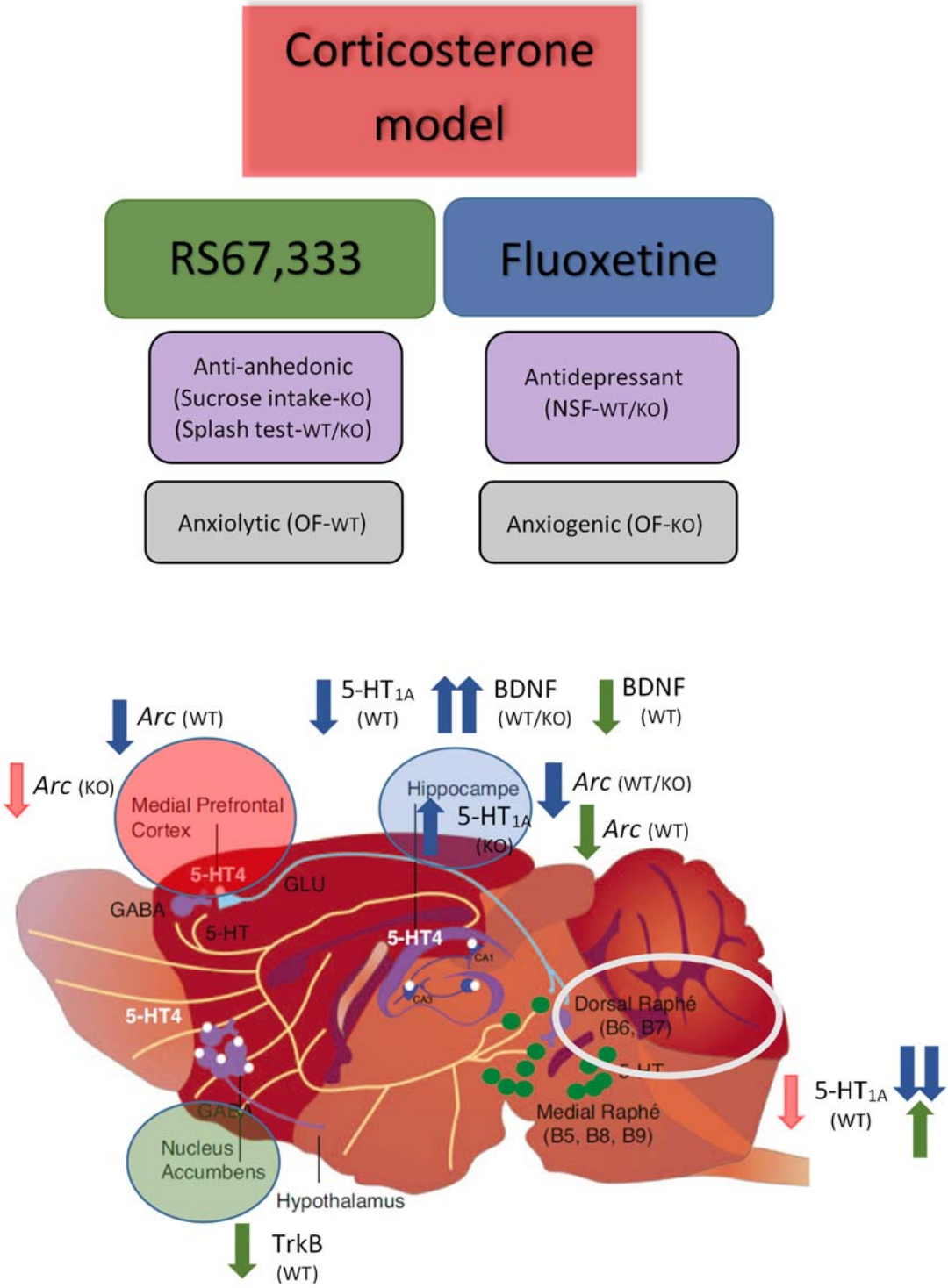


Figure 47. Scheme summarizing the behavioural, neurochemical and molecular changes in WT and 5-HT$_4$ receptor *knockout* mice in the corticosterone model (arrows in red) following chronic administration of RS67,333 (arrows in green) or fluoxetine (arrows in blue).

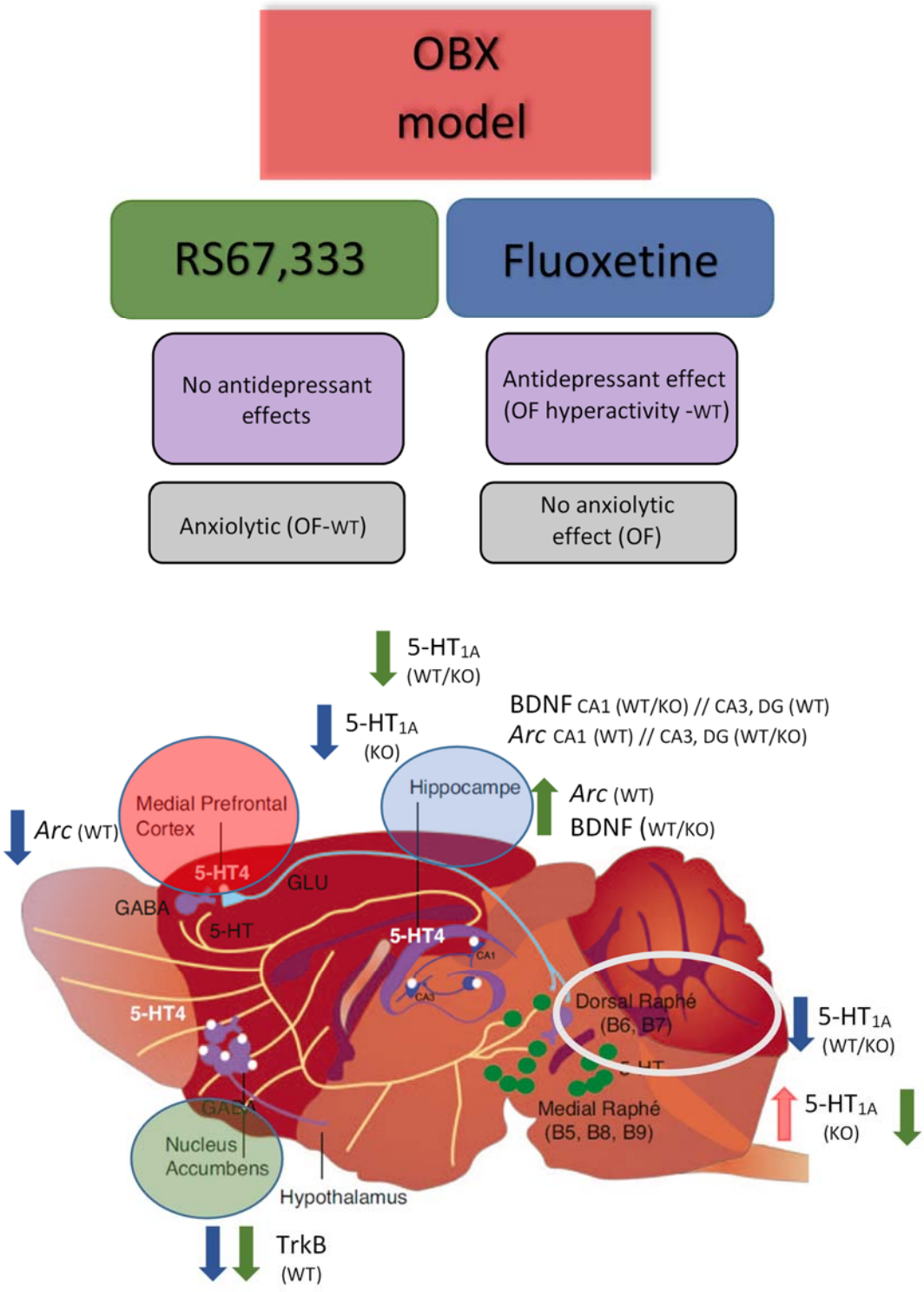


Figure 48. Scheme summarizing the behavioural, neurochemical and molecular changes in WT and 5-HT$_4$ receptor *knockout* mice in the olfactory bulbectomy model (arrows in red) following chronic administration of RS67,333 (arrows in green) or fluoxetine (arrows in blue).

"*La ciencia nunca resuelve un problema sin crear otros 10 más.*"

George Bernard Shaw

VI. Conclusions

1. 5-HT_4 KO mice exhibited "innate" context-dependent anxiety and anhedonia. This behavioural phenotype was associated with a desensitization of 5-HT_{1A} receptors in the dorsal raphe nucleus, as well as an upregulation of BDNF and *Arc* and down-regulation of trkB in cortico-limbic areas. An impaired mTOR signaling, but a "normal" β-catenin signaling, were detected in the hippocampus; however, they showed no changes in hippocampal proliferation.

2. 5-HT_4 receptor KO mice showed the same susceptibility than WT counterparts to the development and the manifestations induced by chronic administration of corticosterone and olfactory bulbectomy.

3. A regulation of the 5-HT_{1A} receptor functionality was detected in both animal models of depression. The more relevant alteration was the desensitization of 5-HT_{1A} autoreceptors in the corticosterone model. A hypersensivity of 5-HT_{1A} receptors was encountered in the frontal cortex in corticosterone-treated KO mice. Olfactory bulbectomy in KO mice was associated with a "normalization" of their innate 5-HT_{1A} autoreceptors desensitization; both genotypes presented a hypersensitivity in hippocampal 5-HT_{1A} receptors.

4. Overall, no relevant changes were observed in BDNF, *Arc*, mTOR and β-catenin following chronic corticosterone or bulbectomy, in both genotypes.

5. RS67,333 exhibited a 5-HT_4 receptor-dependent anxiolytic effect and non-5-HT_4 receptor dependent anti-anhedonic effects. Fluoxetine clearly showed antidepressant actions in both the corticosterone model (5-HT_4 -receptor dependent) and the OBX model (non-5-HT_4 receptor dependent).

6. RS67,333 decreased but fluoxetine increased BDNF mRNA expression in the hippocampus of corticosterone-treated mice. Conversely, RS67,333 increased but fluoxetine decreased BDNF mRNA in cortical areas and the hippocampus of bulbectomized mice.

7. A downregulation in trkB mRNA expression in the nucleus accumbens was encountered, especially after RS67,333 treatment, which could be related to its anti-anhedonic effects in both animal models.

8. The antidepressant effects of fluoxetine were associated with an increased in *Arc* mRNA expression all throughout the brain in both animal models. In contrast, chronic RS67,333 changed *Arc* mRNA expression in very restricted brain areas.

9. Finally, the chronic administration of RS67,333 did not modify mTOR and β-catenin signalling pathways, which could explain, at least in part, its worse behavioural profile than fluoxetine as an antidepressant drug.

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VIII. RESUMEN

INTRODUCCIÓN

La depresión es una de las principales enfermedades neuropsiquiátricas, que afecta entorno al 20% de la población mundial (Hirschfeld, 2012). Se postula que las alteraciones en la transmisión serotonérgica cerebral son la base principal de la depresión, pero también de una amplia mayoría de las enfermedades mentales (Sharp *et al*., 2007). Durante las dos últimas décadas la mayoría de estudios han investigado en profundidad el papel de los receptores 5-HT_1 y 5-HT_2 en la patología depresiva, pero recientemente los receptores 5-HT_4 han ido ganando protagonismo en este campo (Conductier *et al*., 2006; Lucas *et al*., 2007). El análisis de las muestras procedentes de cerebros *postmortem* de sujetos deprimidos demostraron una mayor densidad y funcionalidad de los receptores 5-HT_4 a nivel de la corteza y el estriado (Rosel *et al*., 2004). Mediante la técnica de PET escáner se ha descrito, además, que la disminución de estos receptores en el estriado conlleva un mayor riesgo de sufrir depresión mayor (Madsen *et al*., 2014). Por el contrario, en sujetos sanos tratados con fluoxetina se ha observado una reducción moderada en la densidad de los receptores 5-HT_4 en el estriado y la amígdala (Haahr *et al*., 2014).

A nivel preclínico se ha descrito un incremento en la densidad de receptores 5-HT_4 en el hipocampo ventral y el estriado en dos modelos animales de depresión, como es el caso de la bulbectomía olfatoria (OBX) y los ratones heterocigotos del receptor de glucocorticoides (Licht *et al*., 2010a). Contrariamente, en otro modelo de depresión, como es el caso de las ratas Flinders, se observó una disminución en la densidad del receptor 5-HT_4 a nivel del hipocampo dorsal y ventral (Licht *et al*., 2009).

Los receptores 5-HT_4 también parecen estar implicados en el mecanismo de acción de los fármacos antidepresivos (Lucas *et al*., 2007; Vidal *et al*., 2014). Nuestro grupo de investigación ha descrito una regulación a la baja, tanto de la densidad como de la funcionalidad, de estos receptores en el estriado y el hipocampo de ratas tratadas crónicamente con fluoxetina (Vidal *et al*., 2009) y venlafaxina (Vidal *et al*., 2010). En un estudio más reciente se describió que algunos de los efectos antidepresivos y ansiolíticos de la fluoxetina observados en los paradigmas conductuales predictivos de eficacia antidepresiva/ansiolítica en el modelo animal de corticosterona podrían

estar mediadas por la activación de este receptor (Mendez-David *et al*., 2014). En este contexto cabe mencionar que los receptores 5-HT_4 pueden estar contribuyendo, al menos en parte, a las acciones neurogénicas inducidas por los inhibidores selectivos de la recaptación de serotonina (ISRS) (Imoto *et al*., 2015). En esta misma línea, se ha descrito que el tratamiento a corto plazo con agonistas del receptor 5-HT_4, así como la administración a largo plazo de ISRS, producen un efecto antidepresivo/ansiolítico similar en ratas (Lucas *et al*., 2007, Pascual Brazo *et al*., 2012, Tamburella *et al*., 2009; Vidal *et al*., 2014) asociado con un incremento de la proliferación hipocampal y de los marcadores de plasticidad neural (Pascual-Brazo *et al*., 2012).

La localización anatómica de los receptores 5-HT_4 en el cerebro apoya su participación en la depresión y la ansiedad. Estos receptores se localizan en diferentes estructuras cerebrales del sistema límbico (tubérculo olfatorio, corteza prefrontal, hipocampo, amígdala y el núcleo accumbens) y en los ganglios basales, incluyendo la sustancia negra, donde modulan la liberación de diferentes neurotransmisores entre los que destaca la acetilcolina, la serotonina, el GABA y la dopamina (revisado en Bockaert *et al*., 2011). Desde un punto de vista funcional, los receptores 5-HT_4 situados en la corteza prefrontal medial ejercen un efecto positivo sobre la frecuencia de descarga de las neuronas serotonérgicas del núcleo del rafe dorsal (DRN) (Lucas *et al*., 2005; Lucas y Debonnel, 2002), el principal origen de las proyecciones serotoninérgicas, y cuya actividad se considera crítica para el mantenimiento de un tono serotoninérgico normal.

A pesar de las evidencias descritas anteriormente sobre la implicación de los receptores 5-HT_4 en la depresión y su participación en algunos de los efectos de los fármacos antidepresivos, pocos estudios han investigado las consecuencias conductuales, neuroquímicas y/o moleculares derivadas de la ablación genética de estos receptores. En estudios previos realizados en ratones *knockout* del receptor 5-HT_4 se ha descrito una disminución de la frecuencia de descarga de las neuronas serotonérgicas del núcleo dorsal del rafe e incrementos en el transportador de serotonina, asociados a alteraciones en la densidad de los auto- y heterorreceptores 5-HT_{1A} (Conductier *et al*., 2006). A nivel conductual estos ratones muestran una alteración en su alimentación, en la locomoción, en la ansiedad en respuesta al estrés

y a la novedad, una mayor susceptibilidad a convulsiones y déficits de memoria a largo plazo (Compan *et al*., 2004; Jean *et al*., 2007, Jean *et al*., 2012; Segu *et al*., 2010). A pesar de ello, se desconoce su fenotipo conductual en paradigmas que evalúan respuestas de ansiedad y depresión (por ej. en el *novelty suppressed feeding*, un test basado en un conflicto, o la ingesta de sacarosa que evalúa anhedonia), así como en modelos animales de ansiedad y depresión. También se desconoce el impacto que la delección génica del receptor 5-HT$_4$ pueda tener sobre los procesos neuroplásticos.

Múltiples estudios clínicos y preclínicos demostraron la relevancia de la vía de señalización del factor neurotrófico derivado de cerebro (BDNF) y su receptor TrkB en la fisiopatología y el tratamiento de los trastornos del estado de ánimo (Castrén y Rantamäki, 2010; Duman y Monteggia, 2006). De forma general se puede resumir que en los estudios realizados en animales de experimentación con los fármacos antidepresivos y la terapia electroconvulsiva se observa un aumento de la expresión de BDNF en distintas áreas cerebrales, incluido el hipocampo (Balu *et al*., 2008; Chen *et al*., 2001; Nibuya *et al*., 1995). Similares resultados se obtuvieron en ratas tras la administración subcrónica de un agonista parcial del receptor de 5-HT$_4$ (Pascual-Brazo *et al*., 2012). Además del BDNF y otros marcadores de neuroplasticidad relacionados con la densidad de espinas dendríticas (Peebles *et al*., 2010), se ha relacionado a la proteína *Activity regulated cytoeskeleton* (*Arc*) con la depresión y el mecanismo de acción de los fármacos antidepresivos (De Foubert *et al*., 2004; Li *et al*., 2015).

En función de las evidencias descritas arriba el *objetivo principal* de la presente tesis consiste en clarificar el papel de los receptores 5-HT$_4$ en la depresión y el mecanismo de acción de los fármacos antidepresivos utilizando para ello un modelo genético consistente en la delección génica de este receptor. Para ello nos hemos planteado los siguientes objetivos:

1. Caracterizar el fenotipo a nivel conductual, neuroquímico y molecular de los ratones *knockout* de los receptores 5-HT$_4$.
2. Comparar, en los ratones *wild type* y KO del receptor 5-HT$_4$, el fenotipo a nivel conductual, neuroquímico y molecular en dos modelos animales de

depresión: modelo de la administración crónica de corticosterona y modelo de bulbectomía olfatoria bilateral.

3. Evaluar a nivel conductual, neuroquímico y molecular el efecto del tratamiento crónico con el agonista parcial del receptor 5-HT$_4$ RS67,333 y de la fluoxetina en ambos modelos animales, tanto en ratones *wild type* como en ratones KO del receptor 5-HT$_4$.

MATERIAL Y MÉTODOS

Animales de experimentación

Se utilizaron ratones *knockout* (KO) para el receptor 5-HT$_4$, que fueron donados por Valerie Compan (Compan *et al.,* 2004), con un peso comprendido entre 24-26 gramos y de unos 3 meses de edad. Los ratones KO para el receptor 5-HT$_4$ fueron obtenidos de progenitores heterocigotos o *knockouts* para el receptor 5-HT$_4$ con un *background* 129SvTer y fueron criados en el estabulario de la Universidad de Cantabria. Los animales se estabularon en forma grupal, con un ciclo de luz y oscuridad de 12 horas, y se mantuvieron con libre acceso a la comida y a la bebida, *ab libitum,* exceptuando los experimentos en los cuales se requiere de un período de privación de agua o de comida. Todos los experimentos fueron aprobados por el comité de bioética de la Universidad de Cantabria y fueron realizados de acuerdo con la Legislación Española (Real decreto 53/2013) y la directiva Europea 2010/63/UE.

Diseño experimental

En el primer estudio, se utilizaron ratones *naïve* WT y KO 5-HT$_4$ para llevar a cabo los ensayos de adenilato ciclasa. El segundo estudio tuvo como objetivo comparar el fenotipo a nivel conductual, neuroquímico y molecular de los ratones *naïve* WT y KO 5-HT$_4$. Finalmente, en el tercer estudio, los ratones WT y KO 5-HT$_4$ fueron sometidos a dos modelos animales de depresión/ansiedad: la administración crónica de corticosterona (CORT) y la bulbectomía olfatoria bilateral (OBX) y posteriormente se compararon los efectos del tratamiento con RS67,333 y fluoxetina a nivel conductual, neuroquímico y molecular en cada modelo.

Tratamientos farmacológicos

Los animales se trataron por vía intraperitoneal con el RS67,333 (1,5 mg/kg/día, una vez al día) o su correspondiente vehículo. La fluoxetina se administró por vía oral en el agua de bebida (160 mg/l, equivalente a una dosis de 25 mg/kg/día). Tanto el compuesto RS67,333 como la fluoxetina se administraron durante 14 días en el modelo de corticosterona y 28 días en el modelo de bulbectomía olfatoria. Una vez finalizado el tratamiento los animales se sacrificaron 24 horas después de su última administración.

Ensayo de la actividad de adenilato ciclasa

El ensayo de adenilato ciclasa se realizó para confirmar la falta de funcionalidad de los receptores 5-HT_4 en los ratones KO. Estos ensayos se llevaron a cabo como se ha descrito previamente (Vidal *et al.,* 2009) con ligeras modificaciones. Se homogeneizaron las muestras de tejido estriatal (1:120 w/v) en Tris-HCl 20 mM, EGTA 2 mM, EDTA 5 mM, sacarosa 320 mM, ditiotreitol 1 mM (DTT), leupeptina 25 µg/ml, con la ayuda de un agitador mecánico a 800 rpm. El homogeneizado se centrifugó a 500 x g durante 5 min a 4°C. Los sobrenadantes se centrifugaron de nuevo a 13.000 x g, durante 15 minutos a 4°C, y los sedimentos se resuspendieron en Tris-HCl 20 mM, EGTA 1,2 mM, sacarosa 0,25 M, $MgCl_2$ 6 mM, 3 mM de DTT y 25 µg/mL de leupeptina. Seguidamente, las membranas (25-50 µg de proteína) se preincubaron durante 5 min a 37ºC en el tampón de reacción (Tris-HCl 75 mM pH = 7,4, $MgCl_2$ 5 mM, EGTA 0,3 mM, sacarosa 60 mM, DTT 1 mM, 3-isobutilmetilxantina 0.5mM, fosfocreatina 5 mM, creatina fosfoquinasa 50 U/ml y miocinasa 5 U/ml) y 25 µl de agua (actividad basal) o el agonista del receptor 5-HT_4 zacoprida (10 µM). La reacción se inició mediante la adición de Mg-ATP 0,2 mM y las muestras se incubaron a 37ºC durante 10 min. La reacción se detuvo por ebullición de las muestras durante 4 minutos y luego se centrifugó a 13000 x g durante 5 minutos a 4ºC. La acumulación de AMPc se cuantificó utilizando un kit de ELISA competitivo de AMP cíclico (Thermo Fisher Scientific, Massachussetts, U.S.A.). Las concentraciones de proteína de las membranas se determinaron utilizando el kit de ensayo de proteínas Bio-Rad (Bio-Rad, Munich, Alemania) con BSA como estándar.

Modelos animales de depresión

Modelo de corticosterona. El modelo animal de depresión basado en la administración crónica de corticosterona fue adaptado del método previamente descrito por David *et al*. (2009). Se administró una dosis baja de corticosterona (45 mg/l, equivalente a una dosis de 7-10 mg/kg/día) en el agua de bebida, durante 4 semanas, en botellas opacas y se cambió cada 7 días para evitar la degradación. Después de 4 semanas de tratamiento, se realizó una batería de test de ansiedad y depresión para confirmar el desarrollo del modelo antes del inicio de los tratamientos farmacológicos.

Modelo de bulbectomía olfatoria (OBX). El procedimiento se realizó como se ha descrito previamente por Linge *et al.* (2013) en ratones anestesiados con isofluorano. Tras inyectar lidocaína bajo el cuero cabelludo, se rasuró la cabeza y se realizó una incisión sagital en la línea media. Posteriormente, se realizó una osteotomía (2 mm de diámetro en la línea media 1 mm rostral a la sutura sagital) con la ayuda de un taladro quirúrgico, y a través de este orificio ambos bulbos olfatorios fueron aspirados por una bomba de succión utilizando una cánula de punta roma. A continuación, el orificio se llenó con "cera ósea" con el fin de evitar el sangrado posterior. Finalmente, después de la desinfección de la zona (con solución de yoduro), se cerró el cuero cabelludo con una sutura quirúrgica. Las operaciones de los ratones *sham* se realizaron de la misma manera, aunque los bulbos se dejaron intactos. Tras la cirugía, los animales fueron mantenidos en una sala de postoperatorio (26-27ºC), durante 24-48 horas, antes de ser estabulados en su sala habitual. A continuación, se esperó un período de cuatro semanas antes del inicio de los estudios conductuales para que los ratones se recuperaran y el síndrome de bulbectomía se desarrollara apropiadamente. Al final del estudio, los animales fueron sacrificados y las lesiones fueron verificadas para descartar las lesiones del polo frontal y/o la retirada incompleta de los bulbos olfatorios.

Estudios conductuales

Las pruebas conductuales se realizaron durante la fase de luz y se ordenaron desde la menos a la más estresantes. Los ratones se colocaron en la sala experimental una hora antes del inicio de cada experimento para aclimatarse, con la excepción de los ensayos de ingesta de sacarosa y el test de *nesting*, que se realizaron en su propia jaula en su habitación.

Test del campo abierto. Este test, también conocido como *open field* test, fue realizado siguiendo el protocolo descrito por Amigó *et al*. (2016). El *open field* es una caja de madera (50 cm x 50 cm x 30 cm), en el que el centro (30 cm x 30 cm) está fuertemente iluminado (400 luxes). Los ratones se colocaron en una esquina del *open field*, y se permitió que lo explorasen libremente durante 5 minutos. El comportamiento de los animales fue analizado automáticamente mediante el sistema de *videotracking* del software Any-maze (Stoelting Co., U.S.A.). Se midió la distancia total y la distancia periférica recorridas, así como los parámetros centrales que son indicativos de los niveles de ansiedad de los ratones (tiempo y número de entradas en la zona central).

Test de la caja oscura-caja clara. Este test, también conocido como *light/dark box*, fue realizado siguiendo el protocolo de Clément *et al*. (2009). El aparato está dividido en dos compartimentos de las mismas dimensiones (40 cm x 20 cm x 35 cm) separados por una pequeña puerta. Un compartimento está muy iluminado (400 luxes), mientras que el otro prácticamente no recibe luz. El tiempo y el número de entradas en cada zona fueron registrados y analizados automáticamente mediante el sistema de *videotracking* del software Any-maze (Stoelting Co., U.S.A.).

Test de la supresión de la ingesta inducida por la novedad. Este test, también conocido como *novelty suppressed feeding* (NSF), fue realizado como previamente describió Linge *et al*. (2013). Veinticuatro horas antes del test se les retiró la comida a los ratones dejándoles solo con agua. El día del experimento los ratones se colocaron en el *open field* (50 cm 50 cm x 30 cm) que estaba iluminado tenuemente (40-50 luxes), y que presentaba en el suelo un lecho de serrín. En el centro del *open field* se situó un pellet de comida de unos 2 g aproximadamente. El parámetro

analizado fue la latencia en segundos que tardó el animal en empezar a comer (este parámetro fue medido con la ayuda del software Any-maze) durante un período de hasta 10 minutos. Inmediatamente después de finalizar el test, los animales se colocaron en su jaula y se evaluó el consumo de comida durante un periodo de 5 minutos (consumo de comida post-test).

Test de ingesta de sacarosa. Se realizó siguiendo el protocolo previamente descrito por Linge *et al*. (2013). Los ratones fueron privados de agua durante 24 horas y al día siguiente, se evaluó la cantidad de sacarosa (1%) ingerida por el ratón durante 1 hora.

Test de la natación forzada. Este test, también conocido como *forced swimming test* (FST), fue realizado como describió previamente Porsolt *et al*. (1977). Los ratones fueron introducidos dentro de un cilindro de vidrio (24 cm de alto y un diámetro interno de 12 cm) con agua a una temperatura de 25ºC. Se contabilizó el tiempo de inmovilidad durante los últimos 4 minutos de una sesión de 6 minutos. Se consideró que los animales presentaban *inmovilidad* cuando los ratones estaban flotando, realizando los movimientos mínimos para mantener la cabeza fuera del agua, una *conducta de escalada* cuando realizaban movimientos verticales de entrada y de salida del agua de las patas delanteras y una *conducta de natación* cuando realizaban movimientos de desplazamiento en un plano horizontal. Las tres conductas fueron analizadas por un observador ciego para las condiciones experimentales usando sesiones previamente grabadas del FST.

Test de las canicas. Este test, también conocido como *marble burying test*, fue adaptado del protocolo previamente descrito por Thomas *et al*. (2009). Los ratones se colocaron en una jaula (40 cm x 20 cm x 20 cm) con un lecho de serrín de 5 cm de alto. Encima del lecho, se distribuyeron geométricamente 15 canicas colocadas en 5 filas de 3 canicas. Los ratones exploraron libremente la jaula durante 30 minutos y, transcurrido este tiempo, se contabilizaron el número de canicas enterradas (aquellas que presentaban >50% de su superficie cubierta con el lecho de serrín).

Test del nido o *nesting test.* Fue adaptado del protocolo previamente descrito por Deacon, (2006). El día del test, los ratones fueron estabulados individualmente, y en la jaula se colocó un rectángulo de algodón de unos 5 cm^2 al inicio de la fase oscura. Al cabo de 12 horas, la puntuación de los nidos se evaluó usando la escala de

puntuaciones creada por Deacon, que consta de un rango de 1 a 5 items. En esta escala una puntuación de 1 representa un algodón intacto, o que no se ha producido *nesting*, y una puntuación de 5 corresponde a un nido perfecto.

El ***splash* test**. Fue adaptado a partir del método previamente descrito por David *et al.* (2009). La prueba consistió en aplicar sobre el hocico del ratón un chorro de 200 µl de una solución de sacarosa al 10% lo que provoca un comportamiento de aseo. Inmediatamente después, los ratones se colocaron individualmente en su jaula y su comportamiento se registró durante 5 minutos. El tiempo de *grooming* fue contabilizado manualmente usando los videos pre-grabados.

Evaluación del efecto hipotérmico inducido por (+)-8-OH-DPAT

El protocolo seguido para la medida del efecto hipotérmico inducido por la (+)-8-OH-DPAT fue adaptado del previamente descrito por Zazpe *et al*. (2006). Los experimentos se realizaron en una habitación con control termostático (21 ± 1ºC) entre las 10:00 am y las 14:00 pm. Se evaluó la temperatura corporal durante un periodo de 15 segundos, o hasta que se obtuvo una lectura estable, insertando una sonda termoeléctrica en el recto del animal. Inicialmente, se realizaron tres mediciones a intervalos de 20 minutos considerando el promedio de las dos últimas determinaciones como valor de temperatura basal. A continuación, se inyectó la (+)-8-OH-DPAT (1 mg/kg, i.p.) y se evaluó la temperatura rectal a los 20 minutos y 40 minutos. El efecto de la administración de la (+)-8-OH-DPAT o del vehículo se expresó como cambio de temperatura corporal (ºC, media ± S.E.M.) con respecto a sus valores basales.

Técnicas autorradiográficas

Preparación de los tejidos. Veinticuatro horas después de la última prueba conductual, los ratones se sacrificaron. Sus cerebros se extrajeron, se congelaron inmediatamente sobre hielo seco y posteriormente se almacenaron a -80°C hasta su utilización. El procesado histológico del tejido para los experimentos autorradiográficos se realizó en un microtomo de congelación y consistió en la obtención de secciones tisulares de 14 µm que fueron montadas sobre portaobjetos SuperFrost®plus. Estas secciones se almacenaron a -80ºC en el caso de la hibridación

in situ y a -20ºC en los ensayos de fijación de [^{35}S]GTPγS hasta el día del experimento.

Fijación de [^{35}S]GTPγS inducida por el agonista del receptor 5-HT$_{1A}$. El marcaje del nivel de activación de proteínas G en secciones cerebrales fue realizado según el protocolo previamente descrito por Castro *et al*. (2003a). Las secciones tisulares se preincubaron durante 30 minutos a temperatura ambiente con un tampón que contenía 50 mM Tris-HCl, 0.2 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dl-ditiotreitol and 2 mM GDP (pH= 7,7). Seguidamente, las secciones fueron incubadas, durante 2 horas, en un tampón de la misma composición descrita anteriormente, al cual se añadió adenosina deaminasa (3 mU/ml) junto con [^{35}S]GTPγS (0.04 nM) para determinar la fijación basal. Para determinar la fijación inducida tras la estimulación del receptor 5-HT$_{1A}$, secciones consecutivas a las de los basales fueron incubadas con tampón al cuál se le añadió (±)-8-OH-DPAT (10 μM). La fijación no específica fue determinada en presencia de 10 μM guanosine-5-O-(3-thio)triphosphate (GTPγS). Después de la incubación las secciones se lavaron dos veces, durante 15 minutos, con 50 mM de Tris-HCl (pH= 7,4) a 4ºC, seguido de un lavado en agua fría. Finalmente, las secciones se secaron con una corriente de aire fría, y se expusieron a films Biomax MR junto con los estándares de ^{14}C durante 2 días a 4°C.

Hibridación *in situ*. Las hibridaciones *in situ* se realizaron siguiendo el protocolo descrito por Castro *et al*. (2003b). Las secciones cerebrales se pretrataron antes de iniciar la hibridación *in situ* propiamente dicha. Los tejidos se fijaron con paraformaldehído al 4% en PBS durante 5 minutos y se lavaron dos veces con una solución de PBS. A continuación, se acetilaron con una solución de anhídrido acético al 0,25% en tampón de trietanolamina 0,1 M, durante 10 min, y se deshidrataron en concentraciones crecientes de etanol (70%, 80%, 95% y 100%). Posteriormente se sumergieron en cloroformo durante 10 minutos y, finalmente se rehidrataron con etanol al 100% y 95%. Las secciones se secaron al aire y se almacenaron a -20ºC hasta su uso.

Los oligonucleótidos complementarios al ARNm de BDNF (5'GGTCTC-GTAGAAATATTGGTTCAGTTGGCCTTTTGATACCGGGAC-3'; Vaidya *et al.,* 2001), al ARNm de *Arc* (5' GCAGCTTCAGGAGAAGAGAGGATGGTGC-

TGGTGCTGG-3', Kelly *et al.,* 2008) y al ARNm de TrkB (5'CCTTTCATGCCAAACTTGGAATGTCTCGCCAACTTG-3', Madhav *et al.,* 2001) fueron marcados en el extremo 3' con [^{35}S]dATP usando la desoxinucleótido transferasa terminal (TdT). La sonda marcada (250000 cpm/porta) se mezcló con el tampón de hibridación (formamida desionizada al 50%, citrato salino estándar 4x (SSC), fosfato sódico 10 mM pH = 7,0, pirofosfato de sódico 1 mM, sulfato de dextrano al 10%, solución de Denhardt 5x, ADN de esperma de salmón 200 µg/ml, ácido poliadenílico 100 µg/ml, heparina, 0,12 mg/ml y ditiotreitol 20 mM). Después de la homogeneización, 200 µl de la mezcla se colocaron sobre las secciones tisulares y se incubaron entre 16-18 horas a 42°C en una cámara humidificada. Al día siguiente las secciones se lavaron dos veces, durante 15 minutos a 50ºC, con un tampón SSC 2x conteniendo DTT 1M. Seguidamente se realizaron tres lavados de 5 minutos a temperatura ambiente con SSC 1x, SSC 0,1x y etanol al 80% consecutivamente y finalmente, se lavaron en etanol al 96% durante 1 minuto a temperatura ambiente. Las secciones se secaron al aire y se expusieron a una película BioMax MR a -20ºC durante 2-4 semanas dependiendo de la abundancia del ARNm en el tejido. El control de la especificidad se realizó mediante la incubación de las secciones en presencia un exceso de sonda sin marcar (concentración 1000 veces mayor).

Los autorradiogramas generados se analizaron y cuantificaron utilizando un software de análisis de imágenes Scion Image (Scion Corporation, MD, U.S.A.). Este software permite cuantificar, con niveles de resolución de microscopio óptico, niveles de gris en el área anatómica que se pretende analizar, y los transforma en densidades ópticas. Tanto en la técnica de fijación de [^{35}S] GTPγS como en la hibridación *in situ*, se utilizaron microescalas radioactivas ^{14}C para construir una curva de calibración que relaciona los niveles de radiactividad medidos como nCi/g tejido y la densidad óptica.

Immunohistoquímica de BrdU

La técnica de immunohistoquímica de BrdU se realizó como ha descrito previamente Mostany *et al.* (2008). Las secciones coronales flotantes se incubaron durante 2 h en formamida al 50% / SSC 2x a 65ºC, seguido por una incubación en HCl 2N durante 30 min. Después, las secciones se incubaron durante 10 min en tampón borato 0,1 M

y se lavaron en PBS. Posteriormente, las secciones se incubaron en H_2O_2 al 1% en PBS durante 30 min para inactivar la peroxidasa endógena. Después de varios lavados con PBS, las secciones se incubaron en PBS / 0,2% de Triton X-100/5% de suero de cabra (PBS-TS), durante 30 min, y luego se incubaron con un anticuerpo monoclonal de ratón anti-BrdU (1: 600, ref.: 11170376001 Roche Diagnostics, Barcelona, España) durante toda la noche a 4ºC. La mañana siguiente, después de varios lavados con PBS-TS, las secciones se incubaron durante 2 h con un anticuerpo secundario biotinilado producido en cabra del fragmento Fab de la IgG de ratón (1: 200, ref.: 115-066-006 Jackson Immuno Research Laboratories, Inc., US-PA), seguido de la amplificación con el complejo avidina-biotina (Vector Laboratories). Para la cuantificación de las células $BrdU^+$, se procesó una sección de cada seis a lo largo del hipocampo y se contó bajo un microscopio de luz (Carl Zeiss Axioskop 2 Plus) a una ampliación de 40x y 100x. Se determinó el número total de células $BrdU^+$ por sección y se multiplicó por 6 para obtener el número total de células $BrdU^+$ en el hipocampo.

Western blot

Los ratones fueron sacrificados y sus cerebros fueron extraídos. El hipocampo se diseccionó sobre hielo y se almacenó inmediatamente a -80ºC. El protocolo de extracción de proteínas del hipocampo total fue adaptado de Chomczynski, (1993). Después de la extracción proteica se cargaron 30 µg de proteína para cada muestra en geles SDS-PAGE con una concentración de poliacrilamida del 8.5%. Las condiciones de electroforesis fueron 100 voltios durante 15 minutos seguido por 50 minutos a 160 voltios. Posteriormente, las proteínas se transfirieron a la membrana nitrocelulosa usando un método de transferencia en húmedo y las condiciones de transferencia fueron 100 voltios durante 90 minutos. Después, se bloquearon las uniones inespecíficas de los anticuerpos a las membranas con una solución de leche al 3% que contenía 200 µl de vanadato sódico y 100 µl de fosfato sódico (como inhibidores de la fosfatasa) por cada 100 ml de solución. A continuación, las membranas se incubaron con los anticuerpos primarios durante toda la noche. Se usaron los siguientes anticuerpos primarios: Anti-p-mTOR hecho en conejo(1: 250, Cell signaling Technology, Inc., Massachussets, USA número de referencia # 4517), anti-mTOR hecho en ratón (1: 1000, Cell signaling Technology, Inc. , Massachussets, número USA de referencia 2971), anti-β-catenina hecho en ratón (1: 1000, Santa Cruz

Biotechnology, Texas, EE.UU., número de referencia sc-7963), anti-p-β-catenina hecho en conejo, (Santa Cruz Biotechnology, Texas, EE.UU., número de referencia sc-16743-R), y anticuerpo anti-tubulina hecho en ratón (1: 20.000 Sigma-Aldrich, Missouri, EE.UU., número de referencia T-8660). A la mañana siguiente, se realizaron tres lavados de 15 minutos con TBS-T (150 mM NaCl, 50 mM Tris-HCl/0,05% Tween 20). A continuación, las membranas se incubaron con anticuerpos secundarios anti-conejo y anti-ratón conjugados con un fluoróforo que emite y absorbe en el infrarrojo cercano (1: 15000, LI-COR Bioscience, Nebraska, EE.UU., referencias P/N 925-68072 y P/N 925 - 32213) durante 1 hora. Después de la incubación, se realizaron 3 lavados en TBS-T y se revelaron las membranas utilizando el sistema de imágenes por infrarrojos Odyssey (LI-COR Bioscience, Nebraska, EE.UU.). La cuantificación de las bandas se llevó a cabo mediante densitometría semicuantitativa con el programa informático Image Studio^{TM} Lite (LI-COR Bioscience, Nebraska, EE.UU.). Los valores densitométricos obtenidos con estos anticuerpos se normalizaron con los valores obtenidos con el *house-keeping* tubulina para corregir cualquier posible desviación en la carga de proteínas.

Datos y análisis estadístico. Los datos se representan como media ± E.S.M.. Los análisis estadísticos se realizaron mediante la prueba *t* de Student (para comparar el efecto del genotipo) o un ANOVA de dos y tres vías (con tratamiento, genotipo y modelo como variables), seguida de la prueba post-hoc de Newman-Keuls según proceda. El nivel de significación se estableció en p <0,05. La edición de gráficos y los análisis estadísticos se realizaron utilizando GraphPad Prism 6.0 (La Jolla California EE.UU.) y SPSS 24 (SPSS Inc., Chicago, IL).

RESULTADOS Y DISCUSIÓN

Antes de iniciar los estudios en los ratones *knockout* del receptor 5-HT$_4$ (a partir de este momento nos referiremos a ellos como KO*)* se llevaron a cabo una serie de experimentos de determinación de AMPc para confirmar la ausencia de funcionalidad de dichos receptores en estos ratones. Para este propósito se evaluó el grado de acumulación de AMPc tras la activación del receptor 5-HT$_4$ con el agonista específico zacoprida**.** En los ratones KO, y a diferencia de lo observado en los ratones WT, no

se produjo incremento alguno en la acumulación de AMPc tras la incubación con el agonista.

CARACTERIZACIÓN DE LOS RATONES KO DEL RECEPTOR 5-HT$_4$

ANSIEDAD. Una vez confirmada la ausencia de receptores 5-HT$_4$ funcionales en los ratones KO, evaluamos si la delección genética del receptor 5-HT$_4$ tiene algún impacto sobre el comportamiento ansioso de los ratones. En líneas generales, los ratones KO mostraron una menor actividad central en el *open field*, indicativo de un elevado grado de ansiedad. Este hallazgo contrasta con el comportamiento similar observado entre los ratones WT y KO cuando se evaluaron en el *light/dark box;* sin embargo, hay que tener en consideración que no todos los paradigmas experimentales que evalúan respuestas de tipo ansioso son iguales (Ramos *et al*., 2008). En esta línea se ha descrito la falta de correlación entre el tiempo que un animal permanece en el centro del *open field* y el tiempo que permanece en el compartimento brillante del *light/dark box* (Acevedo *et al*., 2014), lo que corrobora de nuevo que la ansiedad en ambos paradigmas experimentales no tiene que ser parecida. En el test del *novelty suppressed feeding* los ratones de ambos genotipos exhibieron unos niveles de ansiedad similares, reforzando la idea de que la ansiedad evaluada en este paradigma podría ser diferente a la evaluada en las dos pruebas etológicas anteriormente comentadas (Calhoon y Tye, 2015). Finalmente, y en lo que a ansiedad se refiere, al someterlos al test de las canicas observamos que el número de canicas enterradas por los ratones KO fue claramente inferior a la de sus correspondientes WT, lo cual puede interpretarse como una cierta protección frente a la ansiedad. De nuevo, este hecho parece discrepar con lo observado en el test del *open field*, aunque estudios previos ya habían sugerido una ausencia de paralelismo entre los resultados obtenidos en ambas pruebas (Thomas *et al.,* 2009).

DEPRESIÓN. En relación a la influencia de los receptores 5-HT$_4$ en la conducta depresiva, uno de los hallazgos más interesantes es el comportamiento anhedónico que presentan los ratones KO evaluado mediante el consumo de sacarosa. Este estado anhedónico observado en los ratones KO se acompañó de un comportamiento "apático" o "de abandono" (una peor construcción de sus "nidos o lechos") (Filali *et al.,* 2009). Ambos hallazgos refuerzan la hipótesis de que los receptores 5-HT$_4$

podrían estar influyendo en algunas conductas estrechamente relacionadas con el circuito de recompensa. Ambos genotipos exhibieron un comportamiento similar en otros paradigmas que evalúan conductas de tipo depresiva, como son el *forced swimming* test y el *splash* test. Por lo tanto, es importante resaltar que el fenotipo depresivo observado en el test de preferencia de sacarosa y en el "nesting" test es indicativo de que el ratón KO presenta solo ciertas manifestaciones de tipo depresivo.

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Estos resultados observados en los test de conducta sugieren que la delección del receptor 5-HT$_4$ induce una ansiedad innata contexto dependiente y anhedonia.

♦ ♦ ♦

REGULACIÓN DE LA FUNCIONALIDAD DEL RECEPTOR 5-HT$_{1A}$. Seguidamente, y debido al papel que ejercen los receptores 5-HT$_{1A}$ en la conducta ansiosa/depresiva, así como el mecanismo de acción de los fármacos antidepresivos (revisado en Savit *et al.,* 2017), hemos abordado el estudio de la influencia de la delección génica del receptor 5-HT$_4$ sobre la funcionalidad de este receptor. Los estudios *in vitro* en los que se evaluó la funcionalidad del receptor 5-HT$_{1A}$ mediante la técnica de fijación de [^{35}S]GTPγS estimulada por 8-OH-DPAT revelaron una disminución del acople del receptor 5-HT$_{1A}$ a su proteína G en el núcleo dorsal del rafe (NDR) de los ratones KO. Sin embargo, en ambos genotipos se observó un similar grado de hipotermia en respuesta a la inyección del agonista del receptor 5-HT$_{1A}$ 8-OH-DPAT. Resulta sorprendente la aparente discrepancia existente entre los estudios *in vitro* e *in vivo* ya que la hipotermia inducida por 8-OH-DPAT, en ratones, parece depender de la activación de los autorreceptores 5-HT$_{1A}$ (Martin *et al*., 1992; Richardson-Jones *et al*., 2010). Entre las posibles causas cabe destacar que en los estudios *in vivo* pueden producirse mecanismos compensatorios en las vías de señalización intracelular a nivel post-receptorial (por ej. canales Ca^{2+} de tipo GIRK y N / P / Q) que van más allá del acople a las proteínas G y que no puede ser detectada por la técnica *in vitro* utilizada en la presente tesis. Por otra parte, en los estudios *in vitro* hemos observado un aumento en la fijación específica basal de [^{35}S]GTPγS en determinadas áreas del cerebro de los ratones KO. Estos datos parecen reflejar una mayor actividad constitutiva de los receptores acoplados a la proteína G, entre los que se incluye el

receptor 5-HT$_{1A}$, como resultado de un mecanismo compensatorio para contrarrestar la pérdida del receptor 5-HT$_4$.

Un descenso en la funcionalidad del autorreceptor 5-HT$_{1A}$ podría ser responsable del fenotipo ansioso que los ratones KO presentan en el *open field* y, a su vez, podría ser suficiente para mejorar algunas dimensiones de la depresión como son la conducta de "desesperación" pero no otras como la anhedonia. Esta desensibilización "innata" del autorreceptor 5-HT$_{1A}$ podría interpretarse como un mecanismo compensatorio "antidepresivo", paralelo a los cambios que sobre los receptores 5-HT$_{1A}$ del NDR producen los ISRS cuando se administran crónicamente (Castro *et al.,* 2003a; Rosi *et al.,* 2006, 2008).

♦ ♦ ♦

Esta desensibilización de los autorreceptores 5-HT$_{1A}$ puede reflejar una respuesta adaptativa del ratón para contrarrestar la ausencia de la retroalimentación positiva que los receptores 5-HT$_4$ ejercen sobre la frecuencia de descargas de las neuronas serotoninérgicas

♦ ♦ ♦

MARCADORES DE NEUROPLASTICIDAD: BDNF/TRKB Y *ARC*. Numerosos estudios sugieren la implicación del factor neurotrófico (BDNF) y de su receptor trkB en la depresión y en el mecanismo de acción de los antidepresivos (revisado por Duman y Monteggia, 2006; Castrén and Kojima, 2017). Esta vía de señalización de BDNF/trkB también parece estar alterada en determinadas áreas del cerebro, especialmente en el hipocampo, de los ratones KO. Cuando el receptor 5-HT$_4$ está ausente en el cerebro hemos observado un incremento en los niveles de expresión del ARNm que codifica para el BDNF en el giro dentado que se acompaña con una reducción en la expresión del ARNm de su receptor TrkB en otras áreas del hipocampo, la amígdala y el hipotálamo.

Basándose en estos resultados, es tentador hipotetizar la existencia de una relación entre las manifestaciones conductuales de los ratones KO y las alteraciones a nivel molecular. Es posible que la conducta de tipo ansiosa esté asociada al incremento de

BDNF observado en el giro dentado del hipocampo, puesto que, en estudios previos, se ha descrito que la sobreexpresión de BDNF en esta área conduce a un fenotipo de tipo ansioso (Casarotto *et al*., 2012, Deltheil *et al*., 2008 y Deltheil *et al*., 2009). Más difícil es establecer una correlación con los resultados observados en los test de depresión. En los modelos de estrés leve crónico e impredecible (Boulle *et al.,* 2014) y de la bulbectomía olfatoria (Hellweg *et al.,* 2007), un aumento en la expresión del BDNF en el hipocampo se ha asociado la anhedonia; si éste fuese nuestro caso, queda por dilucidar si las alteraciones en la expresión de BDNF son causa o consecuencia. Además de las alteraciones descritas sobre la expresión de BDNF, el ratón KO mostró niveles elevados de la proteína a *Arc* en ciertas áreas corticales e hipocampales. Esta proteína juega un papel relevante en la regulación de la densidad y morfología de las espinas dendríticas y es considerada como una de las dianas de BDNF. Teniendo en cuenta el incremento de BDNF observado en los ratones KO, es posible que esto conlleve a un aumento en la expresión *de Arc,* al menos en el hipocampo. Además, también se han descrito incrementos en la expresión de esta proteína en varios modelos animales de depresión (Boulle *et al.,* 2014; Coppens *et al.,* 2011).

MARCADORES DE NEUROPLASTICIDAD: MTOR/B-CATENINA. En los últimos años ha cobrado gran protagonismo la vía de mTOR como uno de los mecanismos responsables de la eficacia de los fármacos antidepresivos de inicio de acción rápida. Los ratones KO presentan una disminución de la vía de mTOR que está asociada a un comportamiento ansioso y anhedónico. Estos hallazgos concuerdan con estudios previos en los cuales se ha descrito una disminución de la señalización por mTOR en la corteza frontal de pacientes deprimidos (Jerignan *et al*., 2011), así como en el hipocampo de los ratones sometidos a un estrés leve crónico impredecible (Liu *et al*., 2015).

Wnt/β-catenina es una de las vías de señalización intracelular implicada en la proliferación hippocampal (Zhang *et al.,* 2011) y en el mecanismo de acción de los fármacos antidepresivos (Mostany *et al*., 2008; Pilar-Cuellar *et al*., 2014). Nuestros resultados muestran unos niveles de expresión similares de β-catenina y de su forma fosforilada en los ratones *naïve*, WT y KO, lo que refleja un funcionamiento "normal" de esta vía.

Teniendo en cuenta lo descrito anteriormente, no podemos descartar que el aumento de BDNF y/o una señalización normo-funcional de Wnt/β-catenina en el ratón KO constitutivo pueda explicar en parte la ausencia de cambios en la proliferación hipocampal que hemos observado en este ratón.

♦ ♦ ♦

En los ratones KO se observa un incremento en la expresión de BDNF y Arc acompañado de una disminución en la densidad del ARNm del receptor trkB en áreas córtico-límbicas. Asimismo, estos ratones presentan alteraciones en la vía de señalización de mTOR pero no en la de β-catenina a nivel del hipocampo.

♦ ♦ ♦

CARACTERIZACIÓN DE LOS RATONES KO EN LOS MODELOS DE LA ADMINISTRACIÓN CRÓNICA DE CORTICOSTERONA Y LA BULBECTOMÍA OLFACTORIA

ANSIEDAD Y DEPRESIÓN. En relación al modelo animal de la administración crónica de corticosterona (a partir de este momento nos referiremos a él como el modelo de corticosterona), ambos genotipos mostraron un fenotipo ansioso/depresivo similar en todos los paradigmas *(open field, novelty suppressed feding*, test de la sacarosa y *splash test*). Estos hallazgos sugieren que la exposición crónica a la corticosterona parece estar afectando el eje hipotálamo-hipófisis-adrenal con la misma intensidad en ambos genotipos y, por lo tanto, esto explicaría la misma susceptibilidad de los ratones KO a las manifestaciones ansioso/depresivas inducidas por el citado modelo.

De forma similar a lo observado en el modelo anterior, la ausencia constitutiva de los receptores 5-HT_4 no alteró el síndrome inducido por la bulbectomía olfactoria. De hecho, los ratones KO desarrollaron el síndrome típico de este modelo animal de depresión/ansiedad, caracterizado por hiperactividad locomotora, ansiedad y anhedonia, de forma similar al observado en los ratones WT, demostrando la misma susceptibilidad al desarrollo y manifestaciones de dicho modelo.

REGULACIÓN DE LA FUNCIONALIDAD DEL RECEPTOR 5-HT_{1A}. Los dos modelos animales de depresión/ansiedad utilizados en esta tesis regulan la funcionalidad de

los receptores 5-HT$_{1A}$ de forma diferencial dependiendo de su localización a nivel presináptico (autorreceptores) o postsináptico (heterorreceptores).

AUTORRECEPTORES 5-HT$_{1A}$. En el modelo de *corticosterona* se observó una disminución del acople del autorreceptor 5-HT$_{1A}$ a la proteína G exclusivamente en los ratones WT. Esta desensibilización se ha asociado en numerosos estudios a un estado depresivo en humanos (Drevets *et al*., 2000; Drevets *et al*., 2007; Meltzer *et al*., 2004) y en modelos animales de depresión tales como el estrés leve crónico e impredecible (Bambico *et al*., 2009), la privación materna (Leventopoulos *et al*., 2009) y el propio modelo de corticosterona (Hensler *et al*., 2007; Rainer *et al*., 2012). En el caso de los ratones KO la administración de corticosterona no alteró la funcionalidad de los receptores 5-HT$_{1A}$ del núcleo dorsal del rafe, probablemente debido a la desensibilización previa que ya mostraban de base. Independientemente de estos hallazgos, la regulación diferencial que produjo el modelo de corticosterona sobre los autorreceptores 5-HT$_{1A}$ en ambos genotipos no tuvo consecuencias conductuales.

En el modelo de la *bulbectomía olfatoria* se observó una hipersensibilización de los de los receptores 5-HT$_{1A}$ del núcleo dorsal del rafe en los ratones KO cuando se compararon con sus respectivos controles (KO-sham), aunque el grado de estimulación inducida por la 8-OH-DPAT fue similar a la observada en los ratones WT (WT-OBX). Estos datos claramente indican que el proceso de bulbectomía olfatoria produce una "normalización" de la actividad de los receptores 5-HT$_{1A}$ del núcleo dorsal del rafe en los ratones KO que previamente estaban desensibilizados, tal y como se comentó en el apartado del genotipo.

HETERORRECEPTORES 5-HT$_{1A}$. En lo que a los receptores 5-HT$_{1A}$ de localización postsináptica se refiere, la administración de corticosterona no alteró su a funcionalidad en los animales WT, lo que está en consonancia con estudios previos descritos por el grupo de Hensler (Hensler *et al*., 2007); sin embargo, se observó una hipersensibilización de los mismos en la corteza frontal de los ratones KO. A día de hoy no disponemos de una explicación clara de este último hallazgo, máxime cuando la densidad del receptor 5-HT$_{1A}$ no parece estar modificada en la corteza frontal de estos ratones KO (Conductier *et al.,* 2006), por lo que serán necesarias futuras

investigaciones para profundizar en ello. En ambos genotipos se observó una hipersensibilización de los receptores 5-HT_{1A} en el CA3 del hipocampo tras la bulbectomía. Este aumento en la funcionalidad de los receptores 5-HT_{1A} podría deberse a un mecanismo compensatorio en respuesta al descenso en los niveles de serotonina detectados en el hipocampo dorsal en el modelo de bulbectomía (Van der Stelt *et al.,* 2005).

♦ ♦ ♦

Estos resultados demuestran que la ausencia de receptores 5-HT_4 induce una regulación diferencial sobre la funcionalidad de los receptores 5-HT_{1A}, dependiendo del modelo animal de depresión/ansiedad y del genotipo. De todos modos, estos cambios neuroquímicos parecen no tener ningún impacto conductual en las manifestaciones depresivo/ansiosas de los modelos de depresión.

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MARCADORES DE NEUROPLASTICIDAD: BDNF/TRKB Y *ARC*. Los dos modelos animales provocan diversas alteraciones en la expresión de marcadores de neuroplasticidad. El tratamiento crónico con corticosterona no afectó a la expresión del ARNm de BDNF en ambos genotipos, a pesar que los niveles de expresión detectados en los ratones KO (KO-CORT) fueron claramente inferiores a los observados en los ratones WT (WT-CORT) en áreas como la corteza prefrontal medial, el CA1 o el giro dentado del hipocampo. Estos hallazgos, una vez más, sugieren una mayor susceptibilidad de los ratones KO al efecto deletéreo de eventos estresantes. Esta regulación de los niveles de BDNF no se correspondió, en ningún caso, con modificaciones en la expresión del receptor trkB.

Por otro lado, el proceso de extirpación de los bulbos condujo a un incremento en los niveles de expresión de ARNm de BDNF únicamente en la corteza piriforme de ambos genotipos. Esta corteza está implicada en el proceso de reconocimiento olfativo y se ha descrito que la bulbectomía olfatoria podría inducir la diferenciación de las neuronas inmaduras de esta región cerebral (Gómez-Climent *et al*., 2011). Teniendo en cuenta que el BDNF es uno de los factores implicados en la diferenciación neuronal (Chen *et al*., 2013), el incremento de los niveles de este factor

neurotrófico en la corteza piriforme observado en nuestros ratones podría promover dicho proceso de diferenciación. También observamos, en ambos genotipos, un incremento en la densidad del ARN mensajero que codifica para el receptor trkB en el núcleo accumbens de los animales bulbectomizados. Se ha descrito que la infusión bilateral en el núcleo accumbens de antagonistas del receptor trkB provoca respuestas de tipo "antidepresivo" en el modelo de *learned helplessness* (Shirayama *et al.,* 2015), así como en el modelo inflamatorio inducido por la administración del lipopolisacárido (LPS, Zhang *et al.,* 2015). Todo ello nos lleva a postular que el aumento de los niveles de trkB podría estar relacionado con algunas de las manifestaciones de tipo depresivo inducidas por la bulbectomía, en particular la anhedonia.

A diferencia de los cambios observados en la vía de señalización de BDNF/trkB, ninguno de los modelos animales de depresión/ansiedad alteró los niveles de expresión de *Arc.* La ausencia de regulación de esta proteína implicada en procesos de neuroplasticidad es un dato bastante sorprendente en animales que exhiben un fenotipo ansioso/depresivo, debido a la estrecha correlación existente entre *Arc* y algunos de efectos mediados por los fármacos antidepresivos (Li *et al*., 2015). En contraposición, la administración crónica de corticosterona a los ratones KO les produjo una reducción de la expresión de *Arc* en ciertas áreas corticales que podría sugerir una mayor susceptibilidad al modelo, aunque éste no ha sido el caso.

MARCADORES DE NEUROPLASTICIDAD: MTOR Y B-CATENINA. Mientras que la vía de mTOR no parece estar alterada al someter a los ratones a estos modelos animales, si fue el caso para la vía de la β-catenina. En el modelo de corticosterona se detectaron mayores niveles de expresión de β-catenina en el hipocampo de los ratones WT, sin alteraciones en la relación p-β-catenina/β-catenina. Nosotros proponemos que las variaciones en los niveles de GSK-3-β pueden subyacer a este hallazgo, puesto que se ha descrito una mayor actividad fosforiladora de GSK-3-β en ratones tratados crónicamente con corticosterona (Chen *et al*., 2014). Contrariamente, en el modelo de la bulbectomía olfatoria se observó una reducción en la expresión de los niveles de β-catenina en el hipocampo. Estos datos son consistentes con los hallazgos descritos previamente en ratas bulbectomizadas (Rodríguez-Gaztelumendi *et al.,* 2010). Este descenso de los niveles de β-catenina es también consistente con el

aumento de la relación p-β-catenina/β-catenina observada, ya que este hecho, podría acelerar la degradación de la proteína (Ge *et al*., 2016).

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A modo de resumen de este apartado podemos concluir que la ausencia del receptor 5-HT$_4$ no aumenta la vulnerabilidad a la administración crónica de corticosterona ni a la bulbectomía olfatoria bilateral. A pesar de que se encontró una regulación diferencial de los marcadores neuroquímicos y de neuroplasticidad en ambos modelos, éstos no parecen ser suficientes para modificar el fenotipo patológico ansioso/depresivo.

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EFECTO DE LA ADMINISTRACIÓN CRÓNICA DEL RS7,333 Y LA FLUOXETINA EN LOS MODELOS ANIMALES DE DEPRESIÓN

ANSIEDAD Y DEPRESIÓN. El tratamiento crónico con el agonista parcial 5-HT$_4$ RS67,333 en el modelo de corticosterona produjo una respuesta de tipo ansiolítica en el test del ***open field***, únicamente en los ratones WT, que ya fue evidente a la semana de inicio del tratamiento. Este es el primer estudio que describe, en un modelo crónico de depresión/ansiedad, un efecto ansiolítico del RS67,333 dependiente del receptor 5-HT$_4$. Además, hemos confirmado este efecto ansiolítico del RS67,333 en el modelo de bulbectomía olfatoria, aunque se observó a un tiempo más tardío (28 días).

En relación al tratamiento con fluoxetina, después de dos semanas de tratamiento, se observó inesperadamente una conducta ansiosa, evaluada mediante el test del *open field*, en los ratones *naïve* de ambos genotipos. Aunque hoy disponemos de poca literatura sobre ello, es de destacar que recientemente se ha descrito que la fluoxetina tiene un efecto ansiogénico en los ratones *naïve* en el test del *open field* y el laberinto elevado en cruz (Baek *et al*., 2015). Este efecto no se limitó solamente a los ratones *naïve* sino que también se observó ansiedad en los ratones KO tratados con corticosterona cuando fueron tratados, durante 14 días, con el ISRS. Proponemos que esto es debido a la ansiedad "innata" que presentan estos ratones KO y que podría exacerbarse aún más cuando están previamente expuestos a la corticosterona. La

ausencia de efecto de la fluoxetina en los ratones WT tratados con corticosterona está en discrepancia con el efecto ansiolítico descrito en estudios previos (David *et al*., 2009; Mendez-David *et al*., 2014). Esta discrepancia puede explicarse por la mayor duración del tratamiento en dichos estudios (4 *vs* 2 semanas en nuestro estudio). Además, el ISRS carece de efecto ansiolítico en el modelo de bulbectomía olfatoria, lo que claramente refleja que las acciones relacionadas con la ansiedad de la fluoxetina parecen depender no sólo del genotipo, sino también del modelo animal que se considere.

En el ***novelty suppressed feeding*** test, un paradigma de validez predictiva de efecto antidepresivo, el tratamiento crónico (dos semanas) con el RS67,333 no fue capaz de revertir la ansiedad inducida por el modelo de corticosterona. Probablemente se requiere un tiempo de tratamiento más prolongado para detectar una respuesta de tipo ansiolítica/antidepresiva en este test, tal y como describió previamente el grupo de René Hen (Mendez-David *et al*., 2014) a las 4 semanas de administrarle el RS67,333 a una cepa diferente de ratones. En cuanto al tratamiento con la fluoxetina, proporcionamos un nuevo dato sobre el inicio de aparición del efecto de este fármaco antidepresivo en este paradigma experimental. En este estudio demostramos que la fluoxetina revierte la ansiedad inducida por la corticosterona ya a los 7 días de tratamiento, aunque previamente se postuló que se requería al menos de cuatro semanas para lograr tal efecto en el NSF (Dulawa *et al*., 2004; Mendez-David *et al*., 2014, Santarelli *et al*., 2003). Dicho curso temporal se asemejaría al intervalo de tiempo trascurrido entre el inicio de la terapia antidepresiva y la aparición del efecto terapéutico en pacientes con depresión (Fitzgerald, 2014). Sin embargo, este dogma ha sido cuestionado, ya que los resultados de algunos metanálisis demuestran una aparición más temprana del efecto antidepresivo de los ISRS (Posternak *et al*., 2005, Taylor *et al*., 2006), observaciones que van en línea con el resultado que hemos observado en el *novelty suppressed feeding*.

Otro hallazgo novedoso es la misma eficacia ansiolítica/antidepresiva de la fluoxetina observada en los ratones WT y KO en el modelo de corticosterona en el NSF. Este resultado parece estar en desacuerdo con el estudio de Mendez-David *et al*. (2014) en el que demuestra que el bloqueo de los receptores 5-HT_4 revierte los efectos ansiolíticos de la fluoxetina. Es de mencionar que el antagonismo farmacológico no

tiene por qué ser siempre paralelo a lo que observado tras la delección génica, por lo que si depende o no de la presencia de los receptores 5-HT_4 el efecto observado con la fluoxetina en este paradigma experimental es algo que todavía es objeto de debate.

En concordancia con los resultados ya descritos para el RS67,333 en el NSF, este compuesto no revertió la hiperactividad característica de la bulbectomía olfatoria, mientras que la fluoxetina la revirtió de manera tiempo-dependiente. Lucas *et al*., (2007) observó que el RS67,333 revirtió parcialmente la hiperactividad inducida por el modelo de bulbectomía a los tres días de iniciarse el tratamiento, alegando un inicio de aparición rápida del efecto antidepresivo de este agonista. Las diferentes especies utilizadas en ambos estudios (rata en el trabajo de Lucas y ratones en nuestro estudio) podrían explicar esta diferencia. De la misma forma, es posible que algunos efectos del RS67,333 dependan de la cepa de ratón utilizada (Hendriksen *et al*., 2015; Lucki *et al*., 2001). A este respecto comentar que datos de nuestro propio grupo indican que una semana de tratamiento con el RS67,333 produce una reversión de la hiperactividad locomotora de la bulbectomía en la cepa C57/BL6 de ratón (Vidal *et al*., 2013).

Por otro lado, la falta de respuesta observada en los ratones KO bulbectomizados a la fluoxetina, vuelve a resaltar de nuevo el hecho de que la participación de los receptores 5-HT_4 en las manifestaciones conductuales relacionadas con los tests predictivos para valorar una acción ansiolítica/antidepresiva depende del tipo de fármaco, el test utilizado y el modelo animal en el que se evalúe. Esto se deduce claramente en este trabajo al observar los resultados obtenidos con la fluoxetina. En el modelo de bulbectomía olfatoria el ISRS no resultó eficaz en la atenuación de la hiperactividad en los ratones KO, lo que contrasta con los hallazgos obtenidos en el NSF en el modelo de corticosterona. Una posible explicación podría estar relacionada con el papel regulador de los receptores 5-HT_4 en las respuestas motrices, lo que puede contribuir al efecto antidepresivo inducido por la fluoxetina en el modelo de bulbectomía.

Finalmente, y debido a la baja disponibilidad de los ratones KO, hemos evaluado solo el efecto del tratamiento con RS67,333 en la prueba de ingesta de sacarosa y el s*plash test* en ambos modelos animales. La administración crónica del RS67,333 no afectó

a la anhedonia inducida por la corticosterona en los ratones WT. Esta falta de eficacia antidepresiva del RS67,333 fue corroborada posteriormente en los experimentos realizados en el modelo de bulbectomía olfatoria. Estos resultados claramente discrepan con lo publicado en estudios previos en los cuales una semana de tratamiento con el RS67,333 revertió la anhedonia en el modelo animal de estrés leve crónico impredecible (Lucas *et al*., 2007) y en el modelo de corticosterona (Pascual-Brazo *et al*., 2012) en ratas. De nuevo, y a diferencia de la falta de efecto observado del RS67,333 sobre la hiperactividad inducida por OBX en estos ratones, los datos de nuestro propio grupo demuestran que el tratamiento con el RS67,333 revierte la anhedonia inducida por el modelo de bulbectomía en la cepa de ratón C57/BL6 al cabo de una semana de su administración (Vidal *et al.,* 2013). En vista de lo anteriormente expuesto, podemos concluir que las discrepancias entre los resultados obtenidos en esta tesis y a los publicados previamente pueden ser debidas a las diferentes especies usadas (rata *vs* ratón) y/o a las dosis utilizadas (0,75 mg/kg/día en el trabajo de Lucas *vs* 1,5 mg / kg / día en nuestro estudio).

Teniendo en cuenta la falta de eficacia sobre la anhedonia en nuestros ratones WT "deprimidos", el efecto "anti-anedónico" del RS67,333 observado en los ratones KO en la prueba de sacarosa fue totalmente inesperado. No obstante, se ha descrito que el RS67,333 muestra también afinidad por los receptores del subtipo sigma-1 (Eglen *et al*., 1995), cuya activación parece mediar efectos antidepresivos (Fishback *et al*., 2010). Por tanto, podríamos hipotetizar que la ausencia de la principal diana terapéutica del RS67,333 en los ratones KO, favorece la activación de los receptores sigma-1 por este compuesto.

En el *splash test*, un test que evaluaría en ratones una conducta similar a la de "apatía/dejadez" en humanos (Diaz *et al*., 2016; Isigrini *et al*., 2010), el RS67,333 revertió la disminución del tiempo de *grooming* inducida por el modelo de corticosterona en ambos genotipos. Previamente ya se había descrito el mismo efecto para la fluoxetina cuando se administraba durante siete días (Mendez-David *et al*., 2014), por lo que ambos fármacos muestran un patrón temporal similar en este paradigma.

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El RS67,333 muestra, de forma 5-HT$_4$ receptor dependiente, actividad ansiolítica en los modelos animales de depresión y efecto antianhedónico. La fluoxetina posee efecto antidepresivo tanto en el modelo de corticosterona (5-HT$_4$ receptor dependiente) como en el modelo de bulbectomía olfatoria (independiente de la presencia del receptor 5-HT$_4$).

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REGULACIÓN DE LA FUNCIONALIDAD DE LOS AUTORRECEPTORES 5-HT$_{1A}$.

MODELO DE CORTICOSTERONA. El tratamiento crónico con el RS67,333 indujo una regulación de los autorreceptores 5-HT$_{1A}$ opuesta a la regulación producida por la fluoxetina. Es decir, el RS67,333 revierte y la fluoxetina potencia la desensibilización de los receptores 5-HT$_{1A}$ del núcleo dorsal del rafe inducida por la administración crónica de corticosterona. Este es el primer trabajo que describe la regulación de la funcionalidad del receptor 5-HT$_{1A}$ en el modelo de corticosterona tras la administración del RS67,333.

El aumento de la funcionalidad del autorreceptor 5-HT$_{1A}$ observado tras el tratamiento con el RS67,333 en los ratones WT en este modelo podría estar detrás de su falta de eficacia en la prueba del NSF., ya que un incremento en la densidad y funcionalidad del autorreceptor 5-HT$_{1A}$ en ratones induce una reducción de la respuesta a los antidepresivos (Garcia-Garcia *et al*., 2016, Richardson-Jones *et al*., 2010). En este sentido, diversos estudios han descrito que es necesario una activación fisiológica de los autorreceptores 5-HT$_{1A}$ para mantener un estado de ansiedad dentro de la normalidad (Richardson-Jones *et al*., 2011). Este hallazgo podría explicar el efecto ansiolítico del RS67,333 en los ratones WT en el modelo de corticosterona.

Como ya se mencionó anteriormente, el tratamiento crónico con la fluoxetina potenció la desensibilización de los autorreceptores 5-HT$_{1A}$ inducida por la exposición crónica a la corticosterona en los ratones WT. Estos hallazgos están en concordancia con la desensibilización de los receptores 5-HT$_{1A}$ observada *in vivo* en animales tratados crónicamente con fluoxetina en el mismo modelo (Rainer *et al.,*

2012). En lo referente a los animales KO tratados con corticosterona no se ha observado cambios en la funcionalidad de los autorreceptores 5-HT_{1A}; este hecho resalta la dependencia del receptor 5-HT_4 para que se produzca dicha regulación.

MODELO DE BULBECTOMÍA OLFATORIA. El tratamiento crónico con el RS67,333 no indujo cambio alguno, mientras que la fluoxetina produjo una desensibilización de los autorreceptores 5-HT_{1A} en los ratones WT. Este efecto de la fluoxetina no depende de la presencia de los receptores 5-HT_4 puesto que también aparece en los ratones KO.

RATONES NAÏVE. Un resultado sorprendente fue la falta de regulación de los autorreceptores 5-HT_{1A} cuando los ratones WT fueron tratados con fluoxetina durante dos semanas, ya que se había descrito, previamente, una reducción en la capacidad del autorreceptor 5-HT_{1A} para activar la proteína G en ratas durante el mismo tiempo tratamiento (Castro *et al*., 2003a, Hensler, 2002). Probablemente en ratones sea necesario un tiempo de tratamiento más prolongado para que se lleve a cabo dicho proceso. En cuanto al efecto de la fluoxetina en los ratones KO naïve, sabiendo que estos animales muestran una mayor densidad del transportador de serotonina en el DRN (Conductier *et al*., 2006), se esperaría un aumento mayor en los niveles de serotonina en el mesencéfalo lo que conduciría a una mayor desensibilización del autorreceptor. Sin embargo, los ratones KO tratados con fluoxetina exhibieron valores similares de fijación de [^{35}S]GTPγS estimulada por la 8-OH-DPAT a los ratones WT tratados con el vehículo, aunque se observa una tendencia a una mayor funcionalidad en comparación con los valores de los ratones KO tratados con el vehículo (que mostraban una desensibilización de autorreceptor 5-HT_{1A} "innata"). Estos ratones también muestran bajos niveles basales de 5-HT (Conductier *et al*., 2006), por lo que se puede especular que el aumento de los niveles de serotonina inducido por el bloqueo transportador podría ser contrarrestado, dando lugar a niveles de serotonina normales y un normal funcionamiento de los autorreceptores 5-HT_{1A}.

REGULACIÓN DE LA FUNCIONALIDAD DE LOS HETERORRECEPTORES 5-HT_{1A}

La administración crónica del RS67,333, en términos generales, no alteró la funcionalidad de los receptores 5-HT_{1A} postsinápticos en ambos modelos animales,

exceptuando los hallazgos en el CA3 del hipocampo en los ratones bulbectomizados. Sorprendentemente esta regulación no parece estar mediada por los receptores 5-HT_4, un hallazgo que requiere posterior investigación. En relación a los efectos reguladores derivados de la administración crónica de la fluoxetina sobre la sensibilidad de los heterorreceptores 5-HT_{1A}, nuestros hallazgos apuntan a una desensibilización general de los mismos (ej. corteza frontal y el CA1 del hipocampo) en los animales WT, tanto en los ratones sometidos a los modelos animales como en los ratones *naïve*. Es interesante mencionar que esta desensibilización de los receptores observada por la administración de la fluoxetina estaba también presente en el hipocampo de los ratones KO *naïve*. Sin embargo, en los ratones KO tratados con corticosterona, se detectó un incremento significativo en la funcionalidad de los receptores 5-HT_{1A} en el giro dentado tras el tratamiento con el ISRS, lo que parece no ser relevante para los efectos ansiolíticos/depresivos de la fluoxetina en nuestro estudio.

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En resumen, nuestros resultados referentes a la regulación de los receptores 5-HT_{1A} añaden más controversia a este tema, y sugieren que la fluoxetina podría inducir mecanismos adaptativos diferentes de este receptor dependiendo de la especie, del fármaco y de las dosis utilizadas.

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MARCADORES DE NEUROPLASTICIDAD: BDNF

MODELO DE CORTICOSTERONA. El tratamiento crónico con RS67,333 redujo los niveles de expresión de BDNF en el CA1 y el giro dentado en los animales WT sometidos al modelo de corticosterona. En este mismo modelo, y en contraste con lo descrito para el RS67,333, la administración crónica de la fluoxetina produjo una disminución en los niveles de expresión de este factor neurotrófico en el giro centrado, aunque en este caso en ambos genotipos. La regulación opuesta ejercida por ambas sustancias sobre la expresión de BDNF podría ser el sustrato molecular que explica su diferente perfil a nivel conductual: la eficacia antidepresiva de la fluoxetina en el *novelty suppressed feeding* y los efectos diferenciales relacionados sobre la ansiedad en el test del *open field*. Nuestros hallazgos apoyan la hipótesis de que el incremento del

BDNF en el giro dentado, a través de un mecanismo dependiente de la neurogénesis, podría mediar los efectos antidepresivos de la fluoxetina en este paradigma. Además, nosotros hemos encontrado una correlación negativa entre la latencia para comer en el NSF y la densidad de ARNm de BDNF en el giro dentado. Esta correlación se hace todavía más evidente en el caso de los ratones tratados con corticosterona que reciben fluoxetina, resaltando la implicación de esta proteína neurotrófica en el efecto antidepresivo de los ISRS cuando se evalúa en el NSF en condiciones patológicas. Este hallazgo puede explicar, además, la falta de eficacia del RS67,333 en el NSF puesto que, en este caso, no se ha observado una correlación entre ambos parámetros.

A diferencia de lo descrito en los modelos animales, la regulación de la expresión de BDNF parece no ser crítica para el efecto que la fluoxetina ejerce en el NSF en los ratones *naïve*. Por lo tanto, hay que ser cauto a la hora de correlacionar un biomarcador molecular con el efecto antidepresivo de un ISRS, prestando especial atención a si los estudios se realizan en animales *naïve* o en condiciones patológicas, antes de sacar conclusiones traslacionales sobre su efecto terapéutico.

Se ha demostrado que la sobreexpresión del BDNF en el hipocampo podría inducir un comportamiento ansiogénico en el test del *open field* (Deltheil *et al*., 2008; Deltheil *et al*., 2009), y en el *light/dark box* (Casarotto *et al*., 2012), aunque también se ha observado un efecto ansiolítico en el laberinto elevado en cruz (Bahi, 2017). Estas evidencias apoyan la idea de que un aumento de BDNF -como el inducido por el tratamiento con fluoxetina- podría ser perjudicial para observar un efecto ansiolítico en dicho test. Siguiendo el mismo argumento, el descenso en los niveles de BDNF en el hipocampo observados después del tratamiento crónico con el RS67,333, podría ser beneficioso para manifestarse su efecto ansiolítico. En un estudio previo ya se había sugerido el efecto opuesto que el BDNF ejerce sobre algunas conductas ansiosas y depresivas (Govindarajan *et al*., 2006), aunque en este caso el efecto ansiogénico del BDNF se atribuyó al aumento de esta neurotrofina en la amígdala. En nuestro caso podríamos asumir que, de forma similar a lo observado en el hipocampo, el tratamiento crónico con el RS67,333 podría disminuir los niveles de BDNF en la amígdala y, en consecuencia, manifestarse su efecto ansiolítico, tal y como se observa en el test del *open field*. En estudios previos se ha descrito que la fluoxetina incrementa los niveles de ARNm de BDNF en la amígdala (Balu, 2010;

Nowacka *et al*., 2014) lo que podría ser perjudicial para observar un efecto ansiolítico de la fluoxetina en el test del *open field*.

MODELO DE BULBECTOMÍA OLFATORIA. El tratamiento crónico con el RS67,333 y la fluoxetina ejerce una regulación contrapuesta sobre los niveles de expresión de BDNF. El RS67,333 incrementó la expresión de BDNF en áreas corticales (corteza piriforme), mientras que la fluoxetina revertió el incremento en la expresión de BDNF inducida por la bulbectomía olfatoria. A nivel del hipocampo los niveles de expresión de BDNF también se incrementaron y se redujeron tras la administración crónica del RS67,333 y la fluoxetina respectivamente.

Este incremento en los niveles de BDNF observados en los ratones bulbectomizados tratados con el RS67,333 no van asociado a la aparición de una respuesta de tipo "antidepresiva" en los animales, dato que claramente contrasta con la hipótesis neurotrófica. Quizás otro hecho sorprendente es la reducción de los niveles de BDNF en el hipocampo tras la administración crónica de la fluoxetina, asociada a un claro efecto antidepresivo (reversión de la hiperactividad de los ratones sometidos a la bulbectomía olfatoria). En el modelo de bulbectomía olfatoria se ha descrito un aumento de los niveles de BDNF en áreas corticales y del hipocampo de los ratones bulbectomizados (Hellweg *et al*., 2007), proponiéndose que podría tratarse de un mecanismo compensatorio para tratar de contrarrestar el daño de la cirugía. En esta situación la fluoxetina podría abolir la necesidad de la respuesta adaptativa de los mecanismos de neuroplasticidad que produce tras la cirugía (Freitas *et al*., 2013). Si tenemos en cuenta esta suposición, el incremento en los niveles de BDNF tras el tratamiento con el RS67,333 podría estar asociado a la a falta de efecto antidepresivo obsevado con este compuesto. Nuestro estudio refleja la existencia de una correlación positiva entre los niveles de BDNF y la distancia total recorrida en el test del *open field*, lo que refuerza el hecho de que niveles bajos de BDNF hipocampal podría ser beneficioso para revertir la hiperactividad inducida por OBX, como es el caso de la fluoxetina pero no el del RS67,333.

MARCADORES DE NEUROPLASTICIDAD: TRKB. Tanto la administración crónica del RS67,333 como de la fluoxetina produjeron una disminución de la expresión del ARNm que codifica para el receptor trkB en el núcleo accumbens. Este efecto se

observó en los dos modelos animales en el caso del RS76,333 y solamente en el modelo de la bulbectomía en el caso de la fluoxetina. Es importante resaltar este hecho debido a que el núcleo accumbens es una estructura fuertemente implicada en la depresión. Se ha descrito que la infusión de un antagonista del receptor trkB en el núcleo accumbens induce un claro efecto ansiolítico en el test del *open field* en las ratas sometidas a estrés crónico (Azogu y Plamondon, 2017), así como un efecto antidepresivo (Shirayama *et al*., 2015, Zhang *et al*., 2015). Por tanto, la reducción en la señalización mediada por trkB observada después del tratamiento crónico con fluoxetina podría contribuir a la aparición de su efecto antidepresivo.

MARCADORES DE NEUROPLASTICIDAD: *ARC*. En el modelo de corticosterona, el tratamiento crónico con el RS67,333 disminuyó la expresión de *Arc* únicamente en el CA3 del hipocampo, mientras que la fluoxetina parece reducirlo en todo el cerebro, especialmente en el hipocampo. Aunque existe poca información disponible sobre la regulación de *Arc* por fármacos antidepresivos en los modelos animales de depresión/ansiedad, nuestros datos obtenidos con la fluoxetina están en concordancia con lo descrito previamente en el modelo de CUMS (Boulle *et al*., 2014) y el modelo de corticosterona (Monsey *et al*., 2014). En este modelo animal, los efectos moleculares que el tratamiento con la fluoxetina ejerce sobre *Arc, y* también sobre el BDNF, no son dependientes del receptor 5-HT_4. En consecuencia, los efectos conductuales del ISRS que requieren de la modulación de las vías de neuroplasticidad del hipocampo podrían estar todavía presentes en los ratones KO tratados crónicamente con corticosterona.

En relación al modelo de bulbectomía olfatoria, de nuevo nos encontramos con una regulación diferencial de los niveles de *Arc* dependiendo del fármaco administrado. El tratamiento crónico con RS67,333 duplicó los niveles de expresión de *Arc* en el giro dentado de los ratones WT bulbectomizados; mientras que la fluoxetina redujo su expresión en corteza e hipocampo de forma similar a lo observado en el modelo de corticosterona. En el caso del RS67,333 es de resaltar que el incremento de los niveles de *Arc* podría está asociado a su falta de efecto antidepresivo (falta de reversión de hiperactividad característica de la bulbectomía) y en consonancia su falta de efecto sobre la regulación del BDNF.

Finalmente, hemos detectado una regulación diferencial del ARNm de *Arc* por fluoxetina en ratones *naïve* con respecto a lo observado en los modelos de depresión: aumento de su expresión en animales *naïve* y descenso en ambos modelos animales. Nuestros datos en los ratones *naïve* son consistentes con los estudios previos (Alme *et al*., 2007, De Foubert *et al*., 2004, Ferres-Coy *et al*., 2016) y refuerzan la implicación de la neuroplasticidad cerebral en el mecanismo de acción de los fármacos antidepresivos (Duman *et al.,* 2012). También es de destacar que la regulación negativa de *Arc* inducida por la fluoxetina en los modelos animales podría ser un cambio molecular beneficioso, ya que la neuroplasticidad también incluye la eliminación de sinapsis que no median información valiosa (Castrén y Antila, 2017). En este contexto, una reducción de la expresión de *Arc* podría ser beneficiosa para alterar algunas conexiones dañinas que podrían generarse en el estado depresivo, con el fin de modular la funcionalidad aberrante de algunos circuitos cerebrales asociados con los trastornos del estado de ánimo (Cao *et al*., 2014).

MARCADORES DE NEUROPLASTICIDAD: MTOR. El tratamiento crónico con el RS67,333 no alteró la vía de mTOR en ninguno de los grupos experimentales. Se ha descrito que el perfil antidepresivo de la fluoxetina se asocia con una con reversión de la reducción de la fosforilación de mTOR inducida por el modelo de estrés crónico impredecible (Liu *et al*., 2015). Este último hallazgo está en línea con la activación de la vía mTOR que se requiere para observar un efecto antidepresivo tras la estimulación cerebral profunda (Jiménez-Sánchez *et al*., 2016) y la administración de ketamina (Li *et al*., 2010). Por lo tanto, la ausencia de modulación de la señalización mTOR por el RS67,333 podría ser uno de los sustratos moleculares que subyace a los escasos efectos antidepresivos observados con este compuesto en nuestro estudio.

MARCADORES DE NEUROPLASTICIDAD: B-CATENINA. La administración crónica del RS67,333 no afectó a la vía de señalización de β-catenina en los ratones sometidos a cualquiera de los dos modelos animales. La poca eficacia antidepresiva del RS67,333 que hemos observado a lo largo de estudio en los modelos animales de la patología podría deberse también a la falta de regulación de la vía de β-catenina.

♦ ♦ ♦

En conclusión, la regulación de los marcadores de neuroplasticidad observados tras el tratamiento crónico con el RS67,333 se extiende a menos áreas cerebrales y a veces en la dirección opuesta a la modulación observada por la administración crónica de la fluoxetina. Esta regulación opuesta de estas proteínas podría estar detrás de la diferente eficacia en los modelos de depresión.

♦ ♦ ♦

IX. PUBLICATIONS

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The absence of 5-HT$_4$ receptors modulates depression- and anxiety-like responses and influences the response of fluoxetine in olfactory bulbectomised mice: Adaptive changes in hippocampal neuroplasticity markers and 5-HT$_{1A}$ autoreceptor

J. Amigó [a, b, 1], A. Díaz [a, b], F. Pilar-Cuéllar [a, b], R. Vidal [c, d], A. Martín [a], V. Compan [e], A. Pazos [a, b], E. Castro [a, b, *]

[a] Instituto de Biomedicina y Biotecnología de Cantabria, IBBTEC (Universidad de Cantabria, CSIC, SODERCAN), Departamento de Fisiología y Farmacología, Universidad de Cantabria, 39011 Santander, Spain
[b] Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Instituto de Salud Carlos III, Spain
[c] Departamento de Farmacología, Facultad de Medicina, Universidad Complutense, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Spain
[d] Red de Trastornos Adictivos del Instituto de Salud Carlos III, Madrid, Spain
[e] V. Compan University of Nîmes, Site CARMES, 30 000 Nîmes, France

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ABSTRACT

Preclinical studies support a critical role of 5-HT$_4$ receptors (5-HT$_4$Rs) in depression and anxiety, but their influence in depression- and anxiety-like behaviours and the effects of antidepressants remain partly unknown. We evaluated 5-HT$_4$R knockout (KO) mice in different anxiety and depression paradigms and mRNA expression of some neuroplasticity markers (BDNF, trkB and Arc) and the functionality of 5-HT$_{1A}$R. Moreover, the implication of 5-HT$_4$Rs in the behavioural and molecular effects of chronically administered fluoxetine was assessed in naïve and olfactory bulbectomized mice (OBX) of both genotypes. 5-HT$_4$R KO mice displayed few specific behavioural impairments including reduced central activity in the open-field (anxiety), and decreased sucrose consumption and nesting behaviour (anhedonia). In these mice, we measured increased levels of BDNF and Arc mRNA and reduced levels of trkB mRNA in the hippocampus, and a desensitization of 5-HT$_{1A}$ autoreceptors. Chronic administration of fluoxetine elicited similar behavioural effects in WT and 5-HT$_4$R KO mice on anxiety-and depression-related tests. Following OBX, locomotor hyperactivity and anxiety were similar in both genotypes. Interestingly, chronic fluoxetine failed to reverse this OBX-induced syndrome in 5-HT$_4$R KO mice, a response associated with differential effects in hippocampal neuroplasticity biomarkers. Fluoxetine reduced hippocampal Arc and BDNF mRNA expressions in WT but not 5-HT$_4$R KO mice subjected to OBX. These results demonstrate that the absence of 5-HT$_4$Rs triggers adaptive changes that could maintain emotional states, and that the behavioural and molecular effects of fluoxetine under pathological depression appear to be critically dependent on 5-HT$_4$Rs.

1. Introduction

Depression is one of the most prevalent major neuropsychiatric diseases, affecting 20% of the population (Hirschfeld, 2012). Dysfunctions in brain serotonin (5-hydroxytryptamine, 5-HT) volume transmission (Descarries et al., 1975) are postulated to be the major basis of depression, but also of almost all mental diseases (Sharp et al., 2007). During the last two decades, studies have mainly investigated the role of the 5-HT$_1$ and 5-HT$_2$ receptors but, recently,

* Corresponding author. Instituto de Biomedicina y Biotecnología de Cantabria, IBBTEC (Universidad de Cantabria, CSIC, SODERCAN), Avda. Albert Einstein, 22, 39011 Santander, Spain.
E-mail address: castroe@unican.es (E. Castro).
[1] Ph.D. Student in the Departamento de Fisiología y Farmacología, Universidad de Cantabria, 39011 Santander, Spain.

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the 5-HT$_4$ receptors (5-HT$_4$Rs) have taken place in this scenario (Conductier et al., 2006; Lucas et al., 2007). Analyses in *postmortem* brain samples from depressed subjects showed a greater density and functionality of 5-HT$_4$Rs in cortical and striatal areas (Rosel et al., 2004). Moreover, *in vivo* PET imaging studies in humans demonstrated that a reduction in 5-HT$_4$Rs potential binding in the striatum is associated with a high risk to suffer from major depression (Madsen et al., 2014). Conversely, a moderate reduction in the concentration of 5-HT$_4$Rs in both the striatum and amygdala was described in patients treated with fluoxetine for three weeks (Haahr et al., 2014). From the preclinical approach, two different animal models of depression, olfactory bulbectomised (OBX) and glucocorticoid heterozygous receptor mice, showed an increase in the expression of 5-HT$_4$Rs in the ventral hippocampus or striatum, respectively (Licht et al., 2010). In contrast, a down-regulation of 5-HT$_4$Rs in the ventral and dorsal hippocampus was reported in the Flinders-sensitive line rat model of depression (Licht et al., 2009).

The 5-HT$_4$Rs are implicated in the mechanism of action of antidepressants (Lucas et al., 2007; Vidal et al., 2014). We have previously reported a down-regulation of 5-HT$_4$Rs in the striatum and hippocampus of rats chronically treated with fluoxetine (Vidal et al., 2009) and venlafaxine (Vidal et al., 2010). A recent study further described that activation of the 5-HT$_4$Rs may partly mediate some antidepressant and anxiolytic actions of fluoxetine in predictive behavioural paradigms [tail suspension test (TST) for depression and open-field/elevated plus maze tests for anxiety (Mendez-David et al., 2014)]. In this context, it is noteworthy to mention that some of the neurogenic actions induced by selective serotonin reuptake inhibitors (SSRIs) involve the 5-HT$_4$Rs (Imoto et al., 2015). Interestingly at a clinical level, a short-term treatment with a 5-HT$_4$R agonist in rats and long-term administration of SSRIs induced similar antidepressant/anxiolytic actions (Lucas et al., 2007; Pascual-Brazo et al., 2012; Tamburella et al., 2009; Vidal et al., 2014), a behavioural outcome that is associated with an increased hippocampal proliferation and neural plasticity markers (Pascual-Brazo et al., 2012).

The anatomical localization of 5-HT$_4$Rs in the brain supports their involvement in depression and anxiety. These receptors are located in different cerebral structures of the limbic system (olfactory tubercles, prefrontal cortex, hippocampus, amygdala, shell of the nucleus accumbens), the basal ganglia including the substantia nigra (Compan et al., 1996; Waeber et al., 1994), where they modulate the release of different neurotransmitters, including acetylcholine, 5-HT, GABA and dopamine (reviewed in Bockaert et al., 2011). Indeed, the 5-HT$_4$Rs located in the medial prefrontal cortex exert a positive feedback on the firing activity of the dorsal raphe nucleus (DRN) 5-HT neurons (Lucas and Debonnel, 2002; Lucas et al., 2005), the major origin of 5-HT projections and whose activity is admitted to be critical for maintaining a homeostatic brain serotonergic activity. Pharmacological studies have demonstrated that activation of 5-HT$_4$Rs by selective agonists enhances the electrical activity of the DRN 5-HT neurons and, interestingly, chronic administration of 5-HT$_4$R agonists does not induce receptor desensitization in the medial prefrontal cortex (Lucas et al., 2005).

Despite these accumulating evidences about the implication of 5-HT$_4$Rs in depression and in the effects of antidepressants, few studies have investigated the behavioural, neurochemical and/or molecular consequences of the genetic ablation of 5-HT$_4$Rs. A reduced firing (−50%) of the DRN 5-HT neurons, with changes in both the expression of the 5-HT$_{1A}$Rs in the DRN and hippocampus and increased levels of the 5-HT transporter (SERT) and mRNA have been reported in 5-HT$_4$R KO mice (Conductier et al., 2006). Behavioural studies have shown that these mice display abnormal feeding, locomotor and anxiety-like behaviour in response to stress and novelty, seizure susceptibility and long-term memory deficits (Compan et al., 2004; Jean et al., 2007, 2012; Segu et al., 2010). However, whether the 5-HT$_4$R KO mice display specific anxiety- and depression-like behaviours in different contextual situations (*e.g.* novelty suppressed feeding paradigm as a conflict-based test, forced swimming test as a behavioural despair situation, and chronic depression/anxiety models) remains to be fully explored. Similarly, little is known about possible adaptive changes in brain neuroplasticity and neurogenesis in the absence of 5-HT$_4$Rs despite some pharmacological evidences (Imoto et al., 2015; Pascual-Brazo et al., 2012). In this context, brain-derived neurotrophic factor (BDNF)/trkB signalling pathway intervenes in the physiopathology and treatment of mood disorders, as evidenced by clinical and preclinical studies (Castrén and Rantamäki, 2010; Duman and Monteggia, 2006). Animals display increased levels of BDNF following electroconvulsive shock and treatment with classic antidepressant drugs (Balu et al., 2008; Chen et al., 2001; Nibuya et al., 1995), but also when treated with 5-HT$_4$R agonists (Pascual-Brazo et al., 2012). The activity-regulated cytoskeleton associated protein (Arc), and other neuroplasticity markers related to dendritic spine density (Peebles et al., 2010), has also been related to depression and antidepressant drug treatments (De Foubert et al., 2004; Li et al., 2015).

Here, we suspected that mice lacking the 5-HT$_4$Rs could display a depressive- and anxiety-like behaviours, especially in environmental challenges and when subjected to animal models of chronic depression and anxiety. Also we hypothesize that they will show resistance to the behavioural and molecular effects of antidepressants. Therefore, we have performed several behavioural analyses, including fluoxetine treatment in OBX, animal model of chronic depression/anxiety (Linge et al., 2013; Song and Leonard, 2005), in mice lacking 5-HT$_4$Rs. In addition, the functionality of 5-HT$_{1A}$R was evaluated using *in vivo* and *in vitro* techniques because the efficacy of chronic antidepressants is 5-HT$_{1A}$R-dependent (Albert, 2012). Finally, we have extended our analyses by *in situ* hybridization of the BNDF, trkB and Arc mRNA, and hippocampal proliferation.

2. Material and methods

2.1. Animals and experimental groups

The 5-HT$_4$R KO and wild-type (WT) mice (3 months old, 25 ± 1 g) from the breeding of 5-HT$_4$R heterozygote 129SvTer mice (Compan et al., 2004) or 5-HT$_4$R KO mice crossed were housed (n = 4–5 per cage) in the animal house of the University of Cantabria in a temperature − controlled environment with 12 h light/dark cycle, with food and water available *ad libitum*. All experiments were carried out with the approval of the Animal Care Committee of the Universidad de Cantabria and were performed following the Spanish legislation (Real Decreto 53/2013) and the European Communities Council Directive 2010/63/UE on “Protection of Animals Used in Experimental and Other Scientific Purposes”. Before the initiation of the behavioural studies, 5-HT$_4$R stimulated adenylate cyclase assays were performed to ensure the lack of functional 5-HT$_4$Rs in KO mice (see methods and Fig. S1).

Three different sets of animals were used (Fig. S2). The first set of WT and 5-HT$_4$R KO mice were subjected to a battery of anxiety and depression-related tests following a time-schedule (Fig. S2); then, they were sacrificed and their brains used for the *in vitro* studies ([^{35}S]GTPγS autoradiography of 5-HT$_{1A}$R, *in situ* hybridization of BDNF, trkB and Arc, and BrdU immunohistochemistry).

The second set of WT and 5-HT$_4$R KO mice were chronically administered fluoxetine (160 mg/l in the drinking water, equivalent to 25 mg/kg/day) or vehicle (drinking water) for 14 days and tested in the same battery of anxiety and depression-related tests.

The third set of WT and 5-HT_4R KO mice were subjected to bilateral olfactory bulbectomy (OBX) or sham surgery using procedures previously employed in our studies [(Linge et al., 2013, 2016), supplementary material]. After a 4-weeks recovery period, sham and OBX were tested in the open-field to confirm the development of the typical OBX-induced syndrome. Then, OBX mice of both genotypes were administered fluoxetine (160 mg/l in the drinking water, equivalent to 25 mg/kg/day) or vehicle (drinking water) and tested in the open-field at day 14 and 28 of treatment. Finally, they were sacrificed and their brains used for *in situ* hybridization of BDNF and Arc.

2.2. Anxiety and depression tests

Behavioural studies were performed during the light phase, as previously described in detail (Linge et al., 2016). WT and 5-HT_4R KO mice were placed in the experimental room 30 min before the start of each experiment to acclimatize with the exception of the nesting test that was performed during the dark phase with mice placed individually for the session. Behavioural tests were ordered from the least to most stressful one, and leaving an interval between them (usually 2–3 days) to minimize any potential order effects [open-field, light-dark box, sucrose intake, novelty suppressed feeding (NSF) and forced swimming tests (FST)]. Protocols of each test and behavioural testing schedules are described in detail in the supplementary material.

The open-field test was conducted as previously described (Linge et al., 2013, 2016) in order to evaluate the motor reactivity to novelty and anxiety-related parameters (time and distance travelled in the central area).

The light-dark box test was performed as previously described (Clément et al., 2009). Each mouse was initially placed on the dark side of the box and the time and number of entries into each zone were recorded and analysed during 5 min.

The sucrose intake test that represents a “hedonic” index, was performed as previously described (Linge et al., 2016). Mice were deprived of any drink solution for 24 h. The next day, we quantified the amount of consumed sucrose solution (1%) by each animal during 1 h.

The nesting test was performed as previously reported (Deacon, 2006), which evaluates an apathetic and self-neglect behaviour (Pedersen et al., 2014). At the beginning of the dark phase, mice were individually housed and a 3 g piece of cotton was placed inside the cage. The next day, a blind and trained observer scored the nest production according to a 1 to 5 points scale.

The NSF was performed as previously described (Linge et al., 2013). The latency (in seconds) to eat a pellet placed in the centre of the open-field was evaluated following 24 h food deprivation. Food consumption was also evaluated in mice's home-cages (immediately after the NSF test).

The FST permits us to evaluate behavioural despair, as previously described (Porsolt et al., 1977). A blind and trained observer manually scored three behavioural parameters (immobility, swimming, climbing) on video-recorded sessions.

2.3. 8-OH-DPAT-induced hypothermia in mice

The protocol was adapted from Zazpe et al. (2006). The experiments were carried out in a room equipped with a thermostat (21.0 ± 0.5 °C) between 10:00 a.m. and 14:00 p.m. The body temperature was evaluated for a period of 15 s, or until a stable reading was obtained, by inserting a thermoelectric probe into the rectum (room temperature of 20.0 ± 0.1 °C). Initially, three measurements were made at 20 min intervals considering the average of the last two determinations as basal temperature value. Then, 8-OH-DPAT (1 mg/kg) was injected intraperitoneally and the body temperature was evaluated at 20 min.

2.4. In situ hybridization

The brains of mice were rapidly removed and frozen immediately on dry ice and then stored at −80 °C until sectioning. Coronal brain 14 μm thick sections from WT and 5-HT_4R KO mice were cut at −20 °C using a microtome cryostat and thaw-mounted in slices and stored at −20 °C (for [^{35}S]GTPγS binding assay) or −80 °C (for *in situ* hybridization).

The protocol was adapted from Castro (Castro et al., 2003a), using oligonucleotides complementary to BDNF mRNAs 5′-GGTCTCGTAGAAATATTGGTTCAGTTGGCCTTTTGATACCGGGAC-3' (Vaidya et al., 2001) and trkB mRNAs 5′-CCTTTCATGCCAAACTTG-GAATGTCTCGCCAACTTG- 3' (Madhav et al., 2001) and Arc 5′-GCAGCTTCAGGAGAAGAGAGGATGGTGCTGGTGCTGG-3' (Kelly et al., 2008) were 3'end-labelled with [^{35}S]dATP using terminal deoxynucleotide transferase. Finally, 250,000 c.p.m./slide were mixed with hybridization buffer and incubated with brain sections (supplementary materials). The specific distribution of mRNA encoding trkB receptors and BDNF and Arc in the whole brain was consistent with previous studies (Kelly et al., 2008; Madhav et al., 2001; Vaidya et al., 2001).

2.5. [^{35}S]GTPγS autoradiography of 5-HT_{1A}R

Labelling of brain sections (obtained as described above, see 2.4.) with [^{35}S]GTPγS was carried out as previously described (Castro et al., 2003b) in order to evaluate the functionality of 5-HT_{1A}R, using the selective agonist 8-OH-DPAT (10 μM). The non-specific binding was determined in the presence of 10 μM guanosine-5-*O*-(3-thio)triphosphate (GTPγS, supplementary material).

Labelling of coronal brain sections visualized on autoradiograms were analysed and quantified ([^{35}S]GTPγS binding) or semi-quantified (*in situ* hybridization) using a computerized image analysis Scion Image software (Scion Corporation, MD, USA). Optical density values were calibrated using ^{14}C microscales, and expressed in nCi/g of estimated tissue equivalent.

2.6. BrdU-immunohistochemistry

BrdU staining was performed as previously described (Mostany et al., 2008). Free floating coronal sections were incubated 2 h in 50% formamide/2× SSC (saline sodium citrate) buffer at 65 °C, 30 min in 2N HCl, and 10 min in 0.1 M borate buffer. After PBS washing, sections were incubated in 1% H_2O_2 for 30 min, blocked 30 min in PBS/0.2% Triton X-100/5% goat serum and incubated with monoclonal mouse anti-BrdU overnight at 4 °C. After PBS-TS washes, sections were incubated 2 h with biotinylated goat anti-mouse Fab Fragment IgG secondary antibody, followed by amplification with avidin-biotin complex (Vector Laboratories). $BrdU^+$ cells were counted using a light microscope (Carl Zeiss Axioskop 2 Plus) (see supplementary material).

2.7. Drugs and chemicals

[^{35}S]dATP(2′ Deoxyadenosine 5′-(α-thio) Triphosphate, [^{35}S] Guanosine 5′-(γ-thio) Triphosphate (GTPγS), at a specific activity of 1250 Ci/mmol was purchased from Perkin Elmer. Zacopride hydrochloride and fluoxetine hydrochloride were purchased from Tocris Bioscience, and 8-OH-DPAT from Sigma Aldrich. All other chemicals used were of analytical grade.

2.8. Data analysis and statistics

The statistical analyses were performed using Student's *t*-test, Mann-Whitney *U* test or two-way ANOVA. When effects of independent variables (treatment, genotype), or interactions were significant, one-way ANOVAs (treatment, genotype) were performed followed by *post-hoc* test when appropriated. The type of statistical analysis is indicated in the results section and in the legends of figures. The level of significance was set at $p < 0.05$ (Table S1). Graphs editing and statistical analyses were performed using the GraphPad Prism Software (GraphPad, San Diego, CA, USA).

3. Results

3.1. 5-HT$_4$R KO mice display anhedonia and a context-dependent anxiety-like response

In the open-field test, 5-HT$_4$R KO mice presented lower central activity as evidenced by a reduction in the central time (46.7 ± 3.0 s) compared with WT counterparts (60.4 ± 5.5 s, $p < 0.05$, Fig. 1A), with a similar number of entries in the central area (WT: 28.4 ± 1.5 *vs* KO: 24.0 ± 1.9, Fig. 1B). It was not associated with altered locomotion because mice of both genotypes travelled a similar total distance (WT: 20.7 ± 1.5 m *vs* KO: 21.4 ± 0.9 m, Fig. 1C). No difference between the mice of both genotypes was also observed in the LDB (Fig. 1D). Two-weeks treatment with fluoxetine induced a significant reduction of the central time in mice of both genotypes (WT-flx: 35.1 ± 4.6 s *vs* WT, $p < 0.01$; KO-flx: 24.9 ± 6.7 s *vs* KO, $p < 0.01$, Fig. 1A). Accompanied with a significant reduction of the central entries (WT-flx: 17.0 ± 2.0 *vs* WT, $p < 0.01$; KO-flx: 12.9 ± 2.6 *vs* KO, $p < 0.01$, Fig. 1B) but no change was observed in the LDB (Fig. 1D).

Additionally, 5-HT$_4$R KO mice did not show significant changes in the latency to feed following the NSF test (WT: 203.9 ± 35.6 s *vs* KO: 219.7 ± 29.8 s, Fig. 1E). Chronic treatment with fluoxetine induced a similar reduction of the latency to feed in mice of both genotypes (WT-flx: 102.4 ± 11.4 *vs* WT, $p < 0.05$; KO-flx: 118.3 ± 17.9 s *vs* KO, $p < 0.05$, Fig. 1E). Mice of both genotypes consumed a similar amount of food when returned to the home-cage after the NSF test in the basal conditions and following chronic fluoxetine treatment (Fig. 1F).

5-HT$_4$R KO animals showed a lower sucrose intake than WT counterparts (KO: 1.5 ± 0.1 ml *vs* WT: 2.0 ± 0.1 ml, $p < 0.001$, Fig. 2A), an outcome that was reversed by chronic fluoxetine (KO-flx: 2.3 ± 0.1 ml *vs* KO, $p < 0.01$, Fig. 2A). Additionally, an impaired nesting performance was observed in 5-HT$_4$ R KO mice (nesting score of KO: 4.1 ± 0.3 *vs* WT: 4.8 ± 0.1, $p < 0.05$, Fig. 2B). In the FST, mice of both genotypes exhibited similar immobility (WT: 206.4 ± 6.2 s *vs* KO: 204.7 ± 9.1 s, Fig. 2C), swimming (WT: 30.3 ± 5.4 s *vs* KO: 33.1 ± 8.5 s, Fig. 2D) and climbing (WT: 3.4 ± 1.1 s *vs* KO: 2.1 ± 1.1 s, Fig. 2E) scores. Chronic fluoxetine treatment induced similar reductions in immobility (WT-flx: 161.0 ± 12.0 s *vs* WT, $p < 0.01$; KO-flx: 153.3 ± 12.6 s *vs* KO, $p < 0.01$, Fig. 2C), and increases in both swimming (WT-flx: 63.9 ± 9.8 s *vs* WT, $p < 0.05$; KO-flx: 76.1 ± 11.1 s *vs* KO, $p < 0.01$, Fig. 2D) and climbing (WT-flx: 15.1 ± 3.2 s *vs* WT, $p < 0.001$; KO-flx: 11.6 ± 2.4 s *vs* KO, $p < 0.05$, Fig. 2E) behaviours in mice of both genotypes.

3.2. 8-OH-DPAT-induced hypothermia following chronic fluoxetine treatment

The functionality of 5-HT$_{1A}$Rs was assessed *in vivo* by measuring 8-OH-DPAT-induced hypothermia (Fig. 3). A similar decrease of rectal temperature in vehicle-treated mice of both genotypes was observed at 20 min following the administration of 8-OH-DPAT (WT: -2.9 ± 0.3 °C *vs* KO: -2.7 ± 0.3 °C). As expected, chronic treatment with fluoxetine induced a reduction of the hypothermia induced by 8-OH-DPAT administration in mice of both genotypes

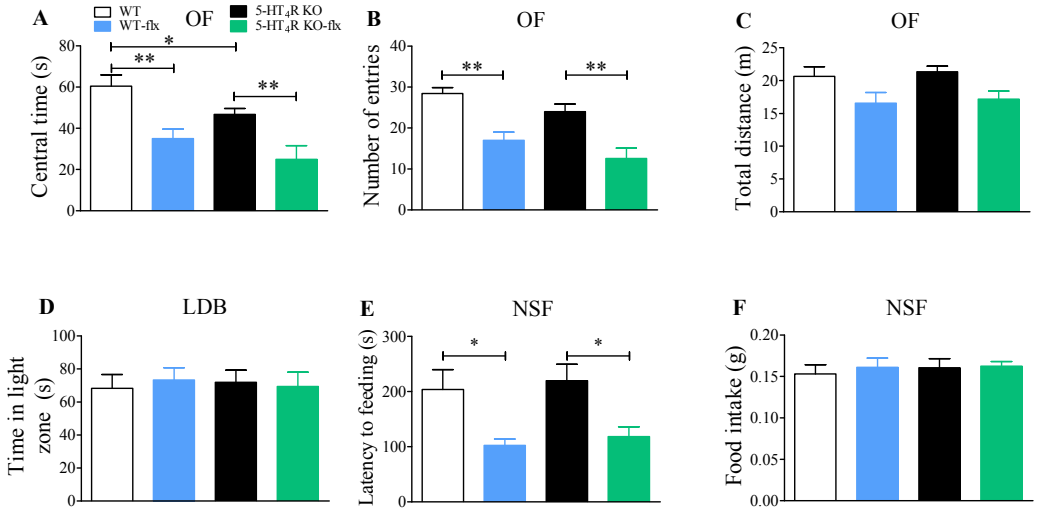

Fig. 1. *Behaviour of WT and 5-HT$_4$R KO mice in different anxiety-related paradigms*. In the open-field test (5 min), 5-HT$_4$R KO mice spent less time than WT counterparts in the central zone, and chronic fluoxetine induced a significant reduction of the central time spent in mice of both genotypes **(A)**. WT and 5-HT$_4$R KO exhibited a similar number of central entries, and chronic fluoxetine induced a similar effect in mice of both genotypes **(B)**. Total distance was not significantly different between mice of both genotypes and following fluoxetine treatment **(C)**. No significant changes were found in the light-dark box test. **(D)** The latency to feed between WT and 5-HT$_4$R KO mice was not different, and a similar reduction was found in mice of both genotypes following the chronic fluoxetine treatment **(E)**. Post-NSF test food intake was not different between mice of both genotypes and following fluoxetine treatment **(F)**. Data are mean ± SEM of $n = 13–18$ mice per group. Two-way ANOVA revealed a main effect of the genotype and treatment on the time spent in the central part of the open-field ($F_{(1,58)} = 6.0$, $p < 0.05$ for genotype effect and $F_{(1,58)} = 23.2$, $p < 0.001$ for treatment effect) **(A)**. Also, a main effect of the genotype ($F_{(1,58)} = 5.1$, $p < 0.05$) and treatment ($F_{(1,58)} = 34.2$, $p < 0.001$) was found on the number of central entries in the open field **(C)** and a treatment effect was found in the latency to feed in the novelty suppressed feeding ($F_{(1,50)} = 14.4$, $p < 0.001$) **(E)**. $^{*}p < 0.05$ and $^{**}p < 0.01$ (Newman-Keuls post hoc test).

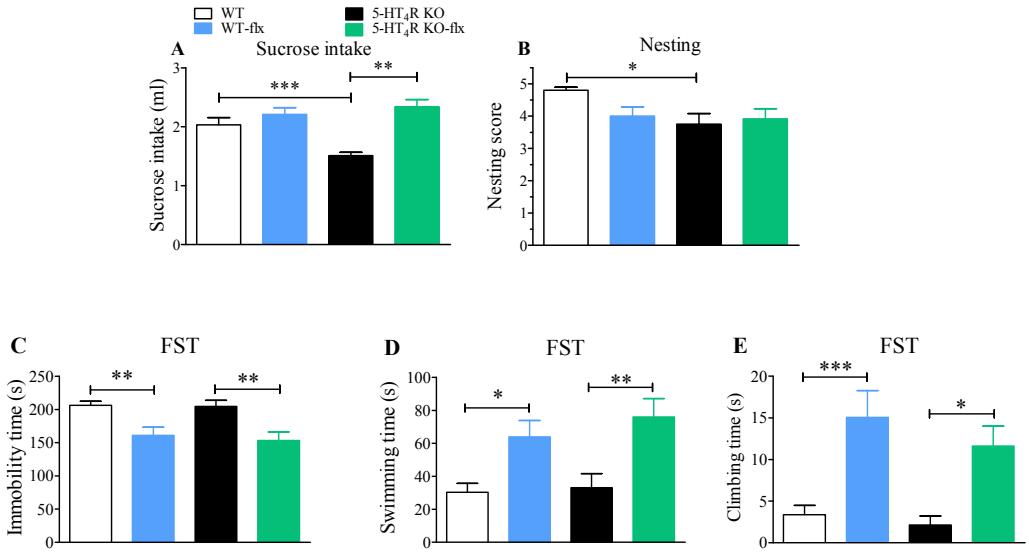

Fig. 2. *Behaviour of WT and 5-HT$_4$R KO mice in different depression-related paradigms.* 5-HT$_4$R KO mice exhibited reduced sucrose intake that was reversed by chronic fluoxetine treatment (**A**), and reduced nesting behaviour (**B**) compared with WT mice. No differences were observed between mice of both genotypes in all FST parameters [immobility (**C**), swimming (**D**) and climbing (**E**)]. In the sucrose intake test, two-way ANOVA analysis revealed a main effect of treatment ($F_{(1,60)} = 22.7$, $p < 0.001$), and a main effect of genotype × treatment interaction ($F_{(1,60)} = 9.5$, $p < 0.01$). In the nesting test, two-way ANOVA analysis revealed a main effect of genotype ($F_{(1,63)} = 4.4$, $p < 0.05$). In the FST, chronic fluoxetine treatment induced similar effects in all the measured outcomes in mice of both genotypes; two-way ANOVA analysis revealed a main effect of treatment (immobility: $F_{(1,51)} = 21.6$, $p < 0.001$; swimming: $F_{(1,51)} = 18.3$, $p < 0.001$; climbing: $F_{(1,50)} = 20.7$, $p < 0.001$). Data are mean ± SEM of $n = 13–20$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Newman-Keuls post hoc test).

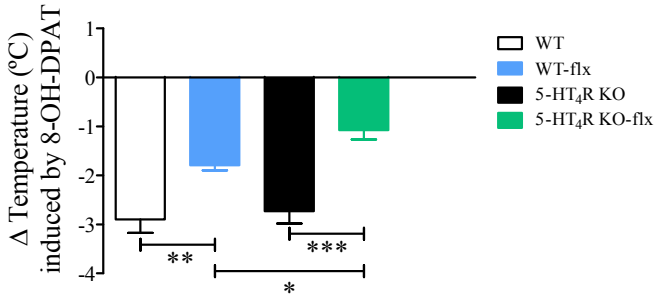

Fig. 3. *Effect of chronic administration of fluoxetine on 8-OH-DPAT-induced hypothermia paradigm.* Chronic administration of fluoxetine induced a reduction of the hypothermic effect of 8-OH-DPAT in mice of both genotypes. Note that 5-HT$_4$R KO mice treated chronically with fluoxetine exhibited a significant lower 8-OH-DPAT-induced hypothermic effect respect to WT counterparts. Two-way ANOVA analysis revealed a main effect of the genotype ($F_{(1,19)} = 4.7$, $p < 0.05$), treatment ($F_{(1,19)} = 42.3$, $p < 0.001$) but not a main effect of the genotype × treatment interaction. Data are mean ± SEM of n = 5–7 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Newman-Keuls post hoc test).

(WT-flx: -1.8 ± 0.1 °C *vs* WT, $p < 0.01$; KO-flx: -1.1 ± 0.2 °C *vs* KO, $p < 0.001$). This reduction was lower in fluoxetine-treated 5-HT$_4$R KO compared with fluoxetine-treated WT mice ($p < 0.05$).

3.3. Reduced G-protein signalling of presynaptic 5-HT$_{1A}$R in 5-HT$_4$R KO mice

The 5-HT$_{1A}$R activity was also assessed *in vitro* by measuring 8-OH-DPAT stimulated [^{35}S]GTPγS binding in brain sections from mice of both genotypes. As shown in Table 1 and Fig. 4, 8-OH-DPAT-induced stimulation of specific [^{35}S]GTPγS binding was lower in the DRN of 5-HT$_4$R KO mice compared with WT counterparts (−28.3%, $p < 0.05$). An increase in basal [^{35}S]GTPγS binding values (nCi/g tissue) was also detected in the brain of 5-HT$_4$R KO mice at the level of both the DRN (WT: 301.8 ± 6.8 *vs* KO: 360.5 ± 19.3, $p < 0.05$) and

Table 1
Absolute values (nCi/g tissue) of specific [^{35}S]GTPγS binding induced by 8-OH-DPAT. DRN: dorsal raphe nucleus, PFrCx: prefrontal cortex, CA1: CA1 field of the hippocampus, CA3: CA3 field of the hippocampus, DG: dentate gyrus of the hippocampus and EntCx: entorhinal cortex. Data are mean ± SEM, number of animals per condition in brackets (n). $^{*}p < 0.05$ (Student's t - test, unpaired data).

Specific [^{35}S]GTPγS binding induced by 8-OH-DPAT (nCi/g tissue)		
Brain areas	WT	5-HT$_4$R KO
DRN	181.9 ± 9.7 (7)	130.4 ± 18.3 (7)*
PFrCx	82.4 ± 17.0 (7)	94.8 ± 19.0 (6)
CA1	194.7 ± 25.8 (7)	222.8 ± 28.7 (7)
CA3	68.7 ± 17.5 (5)	69.4 ± 19.6 (6)
DG	58.8 ± 22.7 (5)	64.4 ± 25.9 (7)
EntCx	167.5 ± 13.5 (6)	218.5 ± 18.8 (7)

the prefrontal cortex (WT: 285.9 ± 25.5 *vs* KO: 377.8 ± 22.4, $p < 0.05$). No significant differences were found either in basal and stimulated [^{35}S]GTPγS binding in the others areas analysed (the hippocampus and entorhinal cortex).

3.4. Altered BDNF, trkB and Arc expression levels in 5-HT$_4$R KO mice

Differences between WT and 5-HT$_4$R KO mice were detected in the levels of both BDNF and trkB mRNA. The highest levels of BDNF and trkB mRNA were observed in the hippocampus of both WT and 5-HT$_4$R KO mice. The 5-HT$_4$R KO mice showed higher increases in the levels of BDNF mRNA in the dentate gyrus (DG) of the hippocampus than WT mice (~35%; $p < 0.05$, Fig. 5A), which was not associated with significant changes in the levels of trkB mRNA (Fig. 5B and F). Additionally, 5-HT$_4$R KO mice exhibited reduced levels of trkB mRNA in the other hippocampal fields (CA1 and CA3: ~15%, $p < 0.01$), and in the amygdala (~26%, $p < 0.05$) compared with WT mice (Fig. 5B and F). No differences were detected in the levels of trkB and BDNF mRNA in the examined areas of the cerebral cortex between mice of both genotypes (Fig. 5A, B and F). Finally, the levels of mRNA encoding Arc (Fig. 5C and F) were increased in the CA1 and CA3 hippocampal fields and the cingulate cortex in 5-

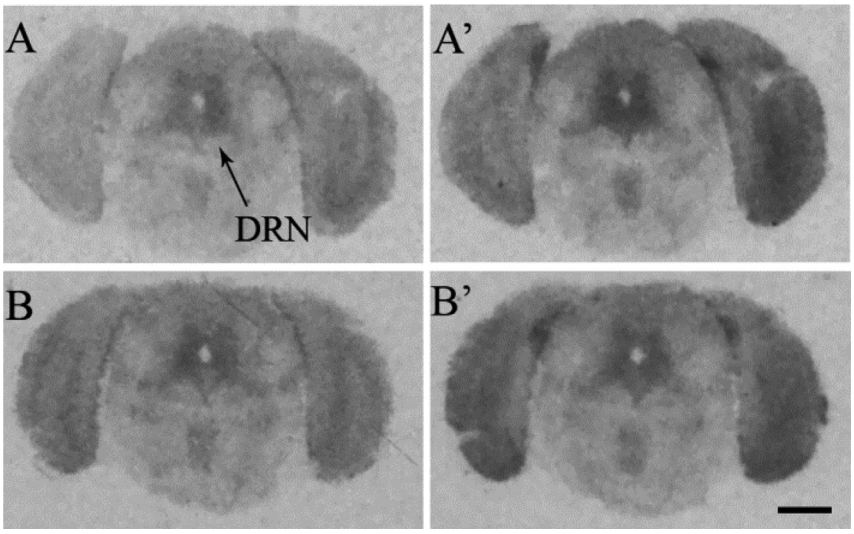

Fig. 4. Autoradiographs in transverse midbrain sections of 8-OH-DPAT stimulated [^{35}S]GTPγS binding. Upper: WT mice, basal (A) and stimulated (A′) binding. Lower: 5-HT$_4$R KO mice, basal (B) and stimulated binding (B′). DRN: dorsal raphe nucleus. Scale bar = 1 mm.

HT$_4$R KO mice (~50%) compared with WT mice ($p < 0.05$).

3.5. Absence of impaired hippocampal proliferation in 5-HT$_4$R KO mice

Hippocampal proliferation was evaluated as the incorporation of the thymidine analogue BrdU in the subgranular zone of the DG. A similar number of BrdU immunolabelled cells was detected in both 5-HT$_4$R KO (1522.0 ± 149.3 BrdU$^+$ cells, Fig. 5E) and WT (1483.0 ± 109.3 BrdU$^+$ cells, Fig. 5D) mice.

3.6. Chronic fluoxetine failed to reverse OBX-induced syndrome in 5-HT$_4$R KO mice

Following four weeks of OBX surgery, mice of both genotypes displayed similar locomotor hyperactivity, as evidenced by the increased total distance travelled in the open-field (WT-sham: 18.3 ± 0.9 m *vs* WT-OBX: 25.4 ± 2.8 m, $p < 0.05$; KO-sham: 16.7 ± 1.0 m *vs* KO-OBX: 24.4 ± 3.2 m, $p < 0.05$, Fig. 6A). A similar temporal pattern of locomotor activity was observed in mice of both genotypes before and after sham or OBX surgery (Fig. S3A and B). This hyperactivity was related to an enhanced thigmotaxis as reflected by an increased ambulation at the periphery of the open-field (WT-sham: 13.8 ± 1.2 m *vs* WT-OBX: 23.6 ± 2.7 m, $p < 0.05$; KO-sham: 12.4 ± 1.2 m *vs* KO-OBX: 22.4 ± 3.1 m, $p < 0.01$) (Fig. S4A).

Mice of both genotypes exhibited similar anxiety-like behaviour induced by OBX, as evidenced by a reduced activity in the central part of the open-field (central time: WT-sham: 49.8 ± 10.3 s *vs* WT-OBX: 9.2 ± 1.4 s, $p < 0.001$; KO-sham: 42.5 ± 6.1 s *vs* KO-OBX: 13.0 ± 3.0 s, $p < 0.001$, Fig. 6B). Similar readouts were observed in other central parameters (Fig. S4B and C).

Considering similarities in OBX-syndrome between both WT and 5-HT$_4$R KO mice, fluoxetine was chronically administrated for 28 days. Animals were again tested in the open-field at days 14 and 28 (Fig. 7). A *post hoc* analysis showed a total reversal of the OBX-induced hyperactivity in fluoxetine-treated WT-OBX mice. Indeed, chronic fluoxetine treatment reduced, in a time-dependent manner, the characteristic OBX-induced locomotor hyperactivity to values similar to those observed in the respective sham-operated mice (WT-OBX-fluoxetine: 10.8 ± 2.4 m *vs* WT-OBX: 23.6 ± 2.7 m, $p < 0.05$) following 28 days of treatment. In contrast, chronic administration of fluoxetine failed to reverse OBX-induced hyperactivity in 5-HT$_4$R KO mice [achieving only 15% of reduction in the total distance travelled, (Fig. 7A)]. Additionally, chronic administration of fluoxetine failed in eliciting a positive effect in the habituation to novelty in 5-HT$_4$R KO mice (Fig. 7B).

3.7. Differential changes in BDNF and Arc mRNA in chronic fluoxetine-treated 5-HT$_4$R KO-OBX mice

In order to set out to explore the neural substrates related to the behavioural outcome of 5-HT$_4$R KO-OBX mice chronically treated with fluoxetine, we assayed the levels of BNDF and Arc mRNA. A differential regulation in plasticity makers was observed between WT and 5-HT$_4$R KO mice. In WT-OBX mice, the chronic fluoxetine treatment induced decreases in the levels of BNDF mRNA in the DG (21%, $p < 0.05$ *vs* WT-OBX, Fig. 8A) and CA3 (31%, $p < 0.05$ *vs* WT-OBX, Fig. 8B) hippocampal areas examined, but not in 5-HT$_4$R KO-OBX mice. The antidepressant exerted a similar effect in the levels of BNDF mRNA in CA1 hippocampal field in mice of both genotypes subjected to OBX (Fig. 8C).

In addition, chronic fluoxetine treatment induced decreases in the levels of Arc mRNA in both WT and 5-HT$_4$R KO-OBX mice in the DG (Fig. 8D) and CA3 (Fig. 8E) hippocampal areas. However, the antidepressant did reduce the levels of Arc mRNA in the CA1 in WT-OBX (56%, $p < 0.01$ WT-OBX-FLX *vs* WT-OBX), but not in 5-HT$_4$R KO mice (Fig. 8F).

4. Discussion

The present study shows that 5-HT$_4$R KO mice display anhedonia and a context-dependent anxiety-like behaviour, with responses to the OBX syndrome similar as those detected in WT mice. A critical present finding is the lack of response of 5-HT$_4$R KO mice to the behavioural and molecular antidepressant effects of fluoxetine in the animal model of chronic depression/anxiety (*e.g.* OBX).

Among all tests used to evaluate the potential depressive-like state of the 5-HT$_4$R KO mice, we detected that these mutant animals consumed less sucrose. It suggests an anhedonic-like behaviour and a specific involvement of 5-HT$_4$Rs in one of the behavioural traits of depression-like behaviour, an outcome reversed by 2-weeks treatment with fluoxetine. Accordingly, the

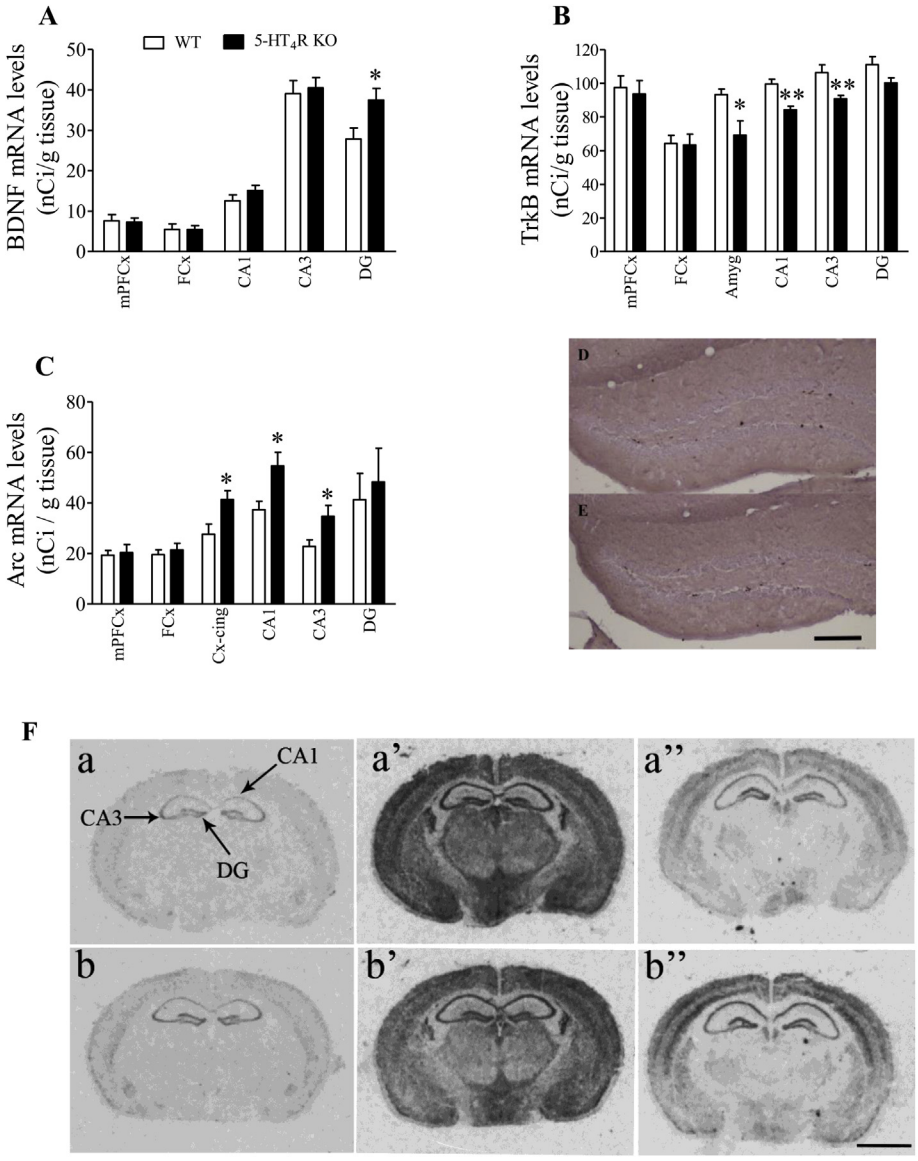

Fig. 5. Changes in neuroplasticity markers in 5-HT$_4$R KO mice. Levels of BDNF (**A**), trkB (**B**) and Arc (**C**) mRNA. Data are mean ± SEM, $n = 6–7$ mice per group. mPFCx: medial prefrontal cortex, FCx: frontal cortex, Amyg: amygdala, CingCx: cingulate cortex, CA1 and CA3: CA1 and CA3 fields of the hippocampus and DG: dentate gyrus. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* WT, Student's *t*-test, unpaired data. Illustrations showing BrdU immunopositive cells in the DG in WT **(D)** and 5-HT$_4$R KO (**E**) mice, scale bar: 20 μm. (**F**) Distribution of BDNF (**a, b**), trkB (**a', b'**) and Arc (**a'', b''**) mRNA visualized on autoradiographs in transverse brain sections from WT (upper) and 5-HT$_4$R KO mice (lower) at the level of the dorsal hippocampus, following *in situ* hybridization. Scale bar: 2 mm.

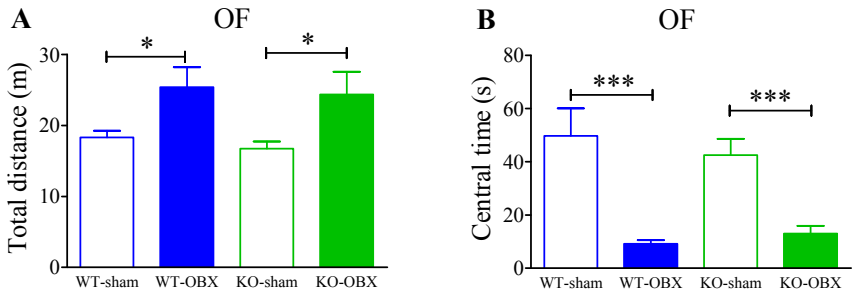

Fig. 6. Similar responses to olfactory bulbectomy in WT and 5-HT$_4$R KO mice. Total distance (**A**) and central time (**B**) in the open-field following 4 weeks post surgery. Data represent mean ± SEM of $n = 7–8$ mice per group. Two-way ANOVA revealed a main effect of the surgery on the total distance travelled ($F_{(1,26)} = 9.4$, $p < 0.01$) and on the time spent in the central part of the open-field ($F_{(1,26)} = 35.1$, $p < 0.001$) but no significant surgery × genotype interaction. $^{*}p < 0.05$ and $^{***}p < 0.001$ (Newman-Keuls post hoc test).

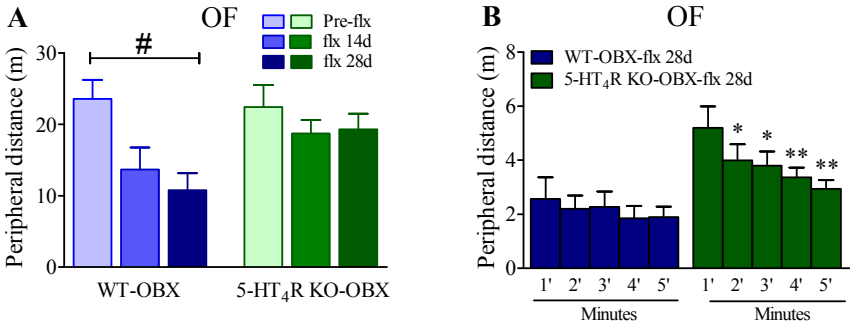

Fig. 7. Chronic fluoxetine failed to reverse OBX syndrome in 5-HT$_4$R KO mice. Total peripheral distance (OF, 5 min session) evaluated before and following fluoxetine (flx) treatment; Two-way ANOVA revealed a genotype × time interaction on the distance travelled at the periphery [$F_{(1,26)} = 7.1$, $p < 0.01$ (**A**)]. Peripheral distance per one min intervals at day 28 of fluoxetine treatment (flx 28d); Two-way ANOVA revealed a significant effect of time ($F_{(4,52)} = 4.7$, $p < 0.01$) and genotype [$F_{(1,13)} = 6.6$, $p < 0.05$ **(B)**]. Data are mean ± SEM of $n = 7–8$ mice per group. $^{\#}p < 0.05$ *vs* pre-flx; $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* 1 min intervals (Newman-Keuls post hoc test). Pre-flx: before the treatment with fluoxetine; flx 14d and flx 28d: 14 and 28 days of fluoxetine treatment.

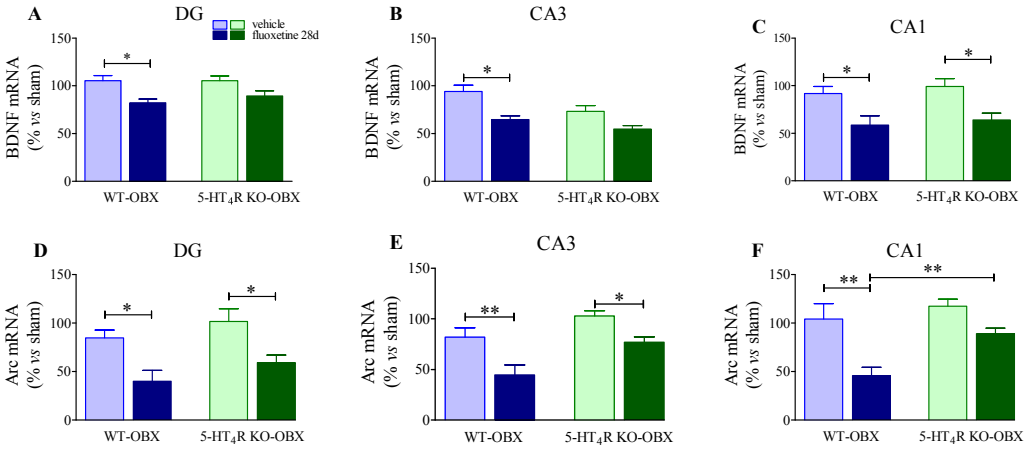

Fig. 8. mRNA expression of neuroplasticity markers in chronic fluoxetine-treated OBX mice. Levels of BDNF (**A, B, C**) and Arc (**D, E, F**) mRNA. Two-way ANOVA analyses revealed a significant main effect of treatment on the levels of BDNF mRNA in the DG ($F_{(1,30)} = 16.1$, $p < 0.001$) and in the CA3 ($F_{(1,31)} = 14.0$, $p < 0.001$) hippocampal areas. Two-way ANOVA analyses also revealed a significant main effect of treatment ($F_{(1,31)} = 13.9$, $p < 0.001$) and genotype ($F_{(1,31)} = 5.9$, $p < 0.05$) on the levels of Arc mRNA in the CA1 hippocampal field. Data are mean ± SEM of $n = 7–8$ mice per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ (Newman-Keuls post hoc test).

mutant mice exhibited a reduction in the nesting score, another behavioural outcome that might reflect both apathetic and anhedonic-like behaviour. However, results in the forced swimming test indicate that 5-HT$_4$R KO mice are not more prone to show higher behavioural despair or learned helplessness than their WT counterparts. These findings appears to be in disagreement with the pharmacological studies reporting a reduced forced swimming test immobility following acute administration of partial 5-HT$_4$R agonists in rats (Lucas et al., 2007). This could be due to (*i*) compensatory neuroplasticity processes that may install gradually over development in the 5-HT$_4$R constitutive KO mice [*e.g.* adaptive changes in serotoninergic system (Conductier et al., 2006), present study], (*ii*) methodological differences (animal species and different FST protocols) and/or (*iii*) because RS67333 is also a partial agonist that could induced different effects depending on the dose used. All this could contribute to the similar response in the FST and also explain the same effect of fluoxetine observed in mice of both genotypes in this experimental paradigm (Cryan et al., 2005). In addition, the differential behaviour of 5-HT$_4$R KO mice in the FST *vs* sucrose/nesting paradigms could be explained by the participation of different brain areas involved in each particular paradigm. In fact, high concentration of 5-HT$_4$Rs has been detected in the shell of the nucleus accumbens in rats and mice (Compan et al., 1996; Jean et al., 2007). There, they intervene in motivation for foods and influence reward processes (Jean et al., 2007, 2012) through the activation of the cAMP/PKA/pCREB pathway (reviewed in Compan et al., 2015). CREB overexpression in the nucleus accumbens reduces the rewarding effects of sucrose (Barrot et al., 2002). And, the ability of cocaine to induce CREB phosphorylation is absent in the nucleus accumbens of the 5-HT$_4$R KO mice (reviewed in Compan et al., 2015), reinforcing the fact that the absence of 5-HT$_4$R favours an anhedonic behaviour (present study). Also, rats subjected to maternal deprivation exhibit a strong correlation between 5-HT$_4$Rs mRNA in the hippocampus and anhedonia-like behaviour (Bai et al., 2014). The absence of 5-HT$_4$Rs in the nucleus accumbens and the hippocampus may likely account for anhedonia-like behaviour of 5-HT$_4$R KO mice. Among the different neuroplasticity markers that have been analysed in the present study, results revealed increased levels of Arc mRNA in the hippocampus and the cingulate cortex of 5-HT$_4$R KO mice. This might support their anhedonia since enhanced expression of Arc mRNA in cortical and hippocampal areas has been described in rodents subjected to social defeat (Coppens et al., 2011) and chronic unpredictable mild stress (Boulle et al., 2014). There is also a reduced concentration in the 5-HT$_{1A}$R in the dorsal hippocampus of 5-HT$_4$R KO mice (Conductier et al., 2006). The participation of these hippocampal 5-HT$_{1A}$Rs in anhedonia and, especially, in the antidepressant effects of fluoxetine must also be considered. Indeed, they may participate in

the anti-anhedonic effect of chronic treatment with fluoxetine observed in 5-HT$_4$R KO mice.

Depression- and anxiety-like behaviours rarely exist independently, and here, in the open-field test, 5-HT$_4$R KO mice presented a reduced central time, suggesting an increased anxiety in good accordance with a previous report (Compan et al., 2004). However, in other tests, which also permit us to evaluate anxiety-like responses under different environmental challenges (light-dark box and novelty suppressed feeding), 5-HT$_4$R KO mice exhibited an anxiogenic response similar to that observed in WT mice. It is well known that different aspects of emotionality are covered by the umbrella term "anxiety" (File, 1992). This discrepancy between the findings in the open-field versus the light-dark box/novelty suppressed feeding tests could be explained when considering the participation of distinct/complementary brain areas that may be differentially engaged in each particular test and/or the particular profile of fluoxetine's effects in anxiety-related paradigm depending on the dose administered (Dulawa et al., 2004). The behavioural findings following chronic fluoxetine treatment accredit this hypothesis since its chronic administration produced opposite effects in the open-field and the novelty suppressed feeding. In fact, fluoxetine induced an anxiogenic effect in the former but a marked anxiolytic effect in the latter test. Moreover, the light-dark box rather than open-field is a more appropriate approach to assess permanent anxiety ["trait anxiety", (File, 1992; Ramos, 2008)], and the novelty suppressed feeding test more reliable evaluation of the mice's performance under a conflictive-aversive context (Belzung and Griebel, 2001). All the above findings suggest that the *Htr*4 gene deficit could enhance anxiety state in a context-dependent manner, but not an anxiety trait, as seen in the 5-HT$_{1A}$R but opposite to 5-HT$_{1B}$R KO mice (Malleret et al., 1999; Ramboz et al., 1998; Zhuang et al., 1999), suggesting a complementary influence of these 5-HT receptors in regulating the different facets of anxiety.

In order to better understand the behavioural phenotype of 5-HT$_4$R KO mice and their response to chronic fluoxetine, we assessed the 5-HT$_{1A}$R functionality by performing *in vivo and in vitro* techniques since this receptor subtype may critically intervene in the efficacy of chronic antidepressant treatments, and in the neurobiology of depression (Albert, 2012).

Similarly to the behavioural outcomes observed, chronic administration of fluoxetine induced a desensitization of 5-HT$_{1A}$Rs in both 5-HT$_4$R KO and WT mice, an outcome already reported in naïve animals treated with this antidepressant (Rainer et al., 2012). This was evidenced by a reduced 8-OH-DPAT-induced hypothermia, though this effect was less apparent in 5-HT$_4$R KO mice, suggesting a higher desensitization of 5-HT$_{1A}$Rs. As discussed below, [^{35}S]GTPγS binding studies demonstrate increased basal binding accompanied with a reduction in 8-OH-DPAT induced [^{35}S]GTPγS binding in the dorsal raphe nucleus. These changes related to the functionality of presynaptic 5-HT$_{1A}$Rs, though not discarding other adaptive mechanisms, may underlie this response of 5-HT$_4$R KO mice in the 8-OH-DPAT-induced hypothermia test after the chronic antidepressant treatment.

Also, 5-HT$_4$R KO mice showed a decreased 8-OH-DPAT-induced stimulation of [^{35}S]GTPγS binding, consistently with a reduced concentration of 5-HT$_{1A}$Rs in the DRN of these mutant mice (Conductier et al., 2006). An increased basal [^{35}S]GTPγS binding was observed in 5-HT$_4$R KO mice, what might be due to a higher constitutive receptor activity, including 5-HT$_{1A}$Rs. If this were the case, it could explain the hypersensitivity of presynaptic 5-HT$_{1A}$Rs, and why citalopram is more efficient to inhibit the firing of 5-HT neurons in 5-HT$_4$R KO mice than in their WT counterparts (Conductier et al., 2006), though this hypothesis requests confirmation. In line with our results in 5-HT$_4$R KO mice, reduced levels of both presynaptic (DRN) and postsynaptic 5-HT$_{1A}$R have been reported in the hippocampus in mice (Conductier et al., 2006), in *postmortem* brain samples from patients with depression (Boldrini et al., 2008; López-Figueroa et al., 2004) and in PET studies (Drevets et al., 2000, 2007; Hirvonen et al., 2008; Meltzer et al., 2004). Animal studies also describe a decline in 5-HT$_{1A}$R expression or functionality in different rodent models of depression/anxiety-following maternal deprivation (Leventopoulos et al., 2009), social defeat (Kieran et al., 2010), chronic unpredictable stress (Bambico et al., 2009) and chronic corticosterone treatment (Rainer et al., 2012). Although it deserves further investigation, these changes on 5-HT$_{1A}$R in the DRN may represent an adaptive response to counterbalance the absence of the positive 5-HT$_4$Rs feedback on the firing activity of DRN serotonergic neurons (Conductier et al., 2006; Lucas and Debonnel, 2002; Lucas et al., 2005).

In using the OBX animal model, we further circumvented how the 5-HT$_4$Rs are potentially involved in some traits of depression- and anxiety-like behaviour, providing a first series of results. As mentioned above and recall here, OBX mediates a depressive-like phenotype as well as other behavioural and neurochemical alterations that can be reversed by chronic antidepressant treatment (Freitas et al., 2013; Linge et al., 2013, 2016; Machado et al., 2012; Song and Leonard, 2005). An earlier study shows an increase in the concentration of 5-HT$_4$Rs in the hippocampus in OBX mice (Licht et al., 2010). However, our study shows that the constitutive absence of 5-HT$_4$Rs did not modify the OBX-induced syndrome. In fact, 5-HT$_4$R KO mice presented a similar behavioural outcome than WT counterparts following OBX (locomotor hyperactivity and anxiety-like behaviour in the open-field, thus showing the same susceptibility to the development and manifestations in this animal model of depression.

The major finding of our study is that chronic fluoxetine was not effective in attenuating OBX-induced hyperactivity in 5-HT$_4$R KO mice, demonstrating its lack of antidepressant effect since the reversal of OBX-induced hyperactivity is meant to have high predictive validity (Freitas et al., 2013; Linge et al., 2013, 2016; Machado et al., 2012; Song and Leonard, 2005). Consistently with our results, a previous study in non-transgenic mice (Mendez-David et al., 2014) showed that, following chronic corticosterone treatment, some anxiolytic/antidepressant effects of fluoxetine are prevented by chronic administration of a selective 5-HT$_4$Rs antagonist. However, as stated above, chronic treatment with fluoxetine induced clear behavioural effects not only in WT but also in 5-HT$_4$R KO mice under basal conditions.

At a molecular level, increased levels of BDNF and Arc mRNA associated with reduced levels of trkB mRNA in non-OBX 5-HT$_4$R KO mice (basal condition) suggest adaptive mechanisms that may likely limit major depressive- and anxiety-like behaviour in these KO mice. Indeed, these molecular factors are well known to influence these behavioural traits (see reviews by Castrén and Rantamäki, 2010; Li et al., 2015). Following OBX surgery, 5-HT$_4$R KO mice treated with fluoxetine did not show the same regulation than WT counterparts in BDNF and Arc expression in the hippocampus. The differences in both the BDNF and Arc mRNA expression detected in the hippocampus of mice of both genotypes could partly underlie the absence of efficacy of fluoxetine in modifying locomotion in OBX-5-HT$_4$R KO mice (present study). Consistently, Freitas et al. (2013) reported that the behavioural effects of chronic fluoxetine in OBX female Swiss mice, are associated with molecular changes (regulation of ERK1/CREB/BDNF) in the hippocampus. Our results suggest that the 5-HT$_4$Rs control of both the BDNF mRNA expression in the DG, and CA3, and Arc mRNA expression in the CA1 can be implicated in these molecular substrates, which can favor the antidepressant effect of fluoxetine. Indeed, Imoto et al. (2015), using 5-HT$_4$R KO mice, introduced a potential role of the 5-HT$_4$Rs in

chronic fluoxetine treatment-induced neurogenic activity and granule cell dematuration in the DG.

Both BDNF and its trkB receptor are implicated in mood disorders (Duman and Monteggia, 2006). Decreased levels of BDNF and trkB mRNA are observed in the hippocampus and frontal cortex in *postmortem* brain samples from patients with depression (Dwivedi et al., 2003; Thompson et al., 2011), and a positive correlation between BDNF serum levels and antidepressant responses was reported in individuals with depression (Brunoni et al., 2008; Sen et al., 2008). Accordingly, chronic stress, a risk factor of major depression, induced a decrease in the expression of BDNF in the hippocampus in animals (Smith et al., 1995). A decreased expression of hippocampal BDNF has been described in the OBX mouse model (Nakagawasai et al., 2016). In contrast, chronic antidepressant treatments (fluoxetine, reboxetine) provoked increases in the levels of BDNF in the hippocampus (Baj et al., 2012). Moreover, BDNF mimics antidepressant-like effects in several behavioural experimental paradigms (Grønli et al., 2006; Murakami et al., 2005). However, the implication of BDNF in anxiety- and depressive-like behaviour is complex and can be contradictory. For instance, reduced BDNF expression in the hippocampus is not associated with a depressive-like phenotype (Taliaz et al., 2010), but with the OBX-depressive behaviour Hendriksen et al., 2012) in rats. Nonetheless, anhedonia and increased levels of BDNF observed in 5-HT_4R KO mice are consistent with the increased hippocampal BDNF expression in mice subjected to chronic unpredictable mild stress (Boulle et al., 2014) and OBX (Hellweg et al., 2007).

This is the first time in which Arc signalling is studied in OBX animals chronically treated with fluoxetine, and the literature on this topic is quite controversial (reviewed in Li et al., 2015). For instance, low levels of Arc mRNA were reported in the frontal cortex and the hippocampus following chronic social isolation stress in mice (Ieraci et al., 2016), but increased levels were found in rats following social defeat (Coppens et al., 2011) and in mice subjected to chronic unpredictable mild stress (Boulle et al., 2014). Pharmacological studies have reported that chronic SSRI treatment stimulates Arc mRNA expression in the cingulate and orbital frontal cortices in rats without producing any change in the hippocampus (De Foubert et al., 2004), and that chronic treatment with agomelatine normalized CUMS-induced increases in the levels of Arc mRNA in the hippocampus (Boulle et al., 2014). It can be speculated that the increased levels in Arc mRNA due to the absence of 5-HT_4Rs could represent a compensatory mechanism for the life-long loss of 5-HT_4Rs. It has been reported that 5-HT_4R KO mice exhibit an increased muscarinic neurotransmission (Segu et al., 2010), which may account for the increased levels of Arc (and BDNF). Indeed, a direct relationship between cholinergic transmission and these neuroplasticity proteins has been reported regarding spatial memory acquisition (Gil-Bea et al., 2011).

In conclusion, our study shows that the absence of 5-HT_4Rs modulates the response of mice in depression- and anxiety-like experimental paradigms and did not influence the behavioural effects of chronic fluoxetine treatment. However, fluoxetine failed to reverse OBX-induced syndrome in 5-HT_4R KO mice, a response classically associated with differential effects in hippocampal neuroplasticity biomarkers. These results demonstrate that the absence of 5-HT_4Rs triggers adaptive changes that could maintain a global adaptive emotional state with the exception of anhedonia and a context-dependent anxiety. These findings further unmask that the behavioural and molecular effects of fluoxetine under pathological depression appear to be critically dependent on 5-HT_4Rs.

Disclosure

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.08.037.

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