Can we increase the speed and efficacy of antidepressant treatments?

Part II. Glutamatergic and RNA interference strategies

Artigas F^{1, 2, 3}, PhD; Celada P^{1, 2, 3}, PhD; Bortolozzi A^{1, 2, 3}, PhD

¹Department of Neurochemistry and Neuropharmacology, Institut d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas (CSIC), Spain

²CIBERSAM (Centro de Investigación Biomédica en Red de Salud Mental), Spain

³Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Spain

Corresponding author: Francesc Artigas, PhD; Dept. of Neurochemistry and Neuropharmacology, IIBB-CSIC (IDIBAPS), Rosselló, 161, 6th floor, 08036 Barcelona, Spain. Phone: +3493-363 8314; Fax: +3493-363 8301; e-mail: francesc.artigas@iibb.csic.es

Running title. Antidepressant improvement: Glutamatergic and RNAi strategies

Abstract. In the second part we focus on two treatment strategies that may overcome the main limitations of current antidepressant drugs. First, we review the experimental and clinical evidence supporting the use of glutamatergic drugs as fast-acting antidepressants. Secondly, we review the involvement of microRNAs in the pathophysiology of major depressive disorder (MDD) and the use of small RNAs (e.g., small interfering RNAs or siRNAs) to knockdown serotonergic genes and induce antidepressant-like responses in experimental animals.

The development of glutamatergic agents is a promising venue for antidepressant drug development, given the antidepressant properties of the non-competitive NMDA receptor antagonist ketamine. Its unique properties appear to result from the activation of AMPA receptors by a metabolite [(2S,6S;2R,6R)-hydroxynorketamine (HNK)] and mTOR signaling. These effects increase synaptogenesis in prefrontal cortical pyramidal neurons and enhance serotonergic neurotransmission via descending inputs to the raphe nuclei. This view is supported by the cancellation of ketamine's antidepressant-like effects by inhibition of serotonin synthesis.

We also review existing evidence supporting the involvement of microRNA (miRNA) in MDD and the preclinical use of RNA interference (RNAi) strategies to target genes involved in antidepressant response. Many miRNAs have been associated to MDD, some of which –e.g., miR-135- target genes involved in antidepressant actions. Likewise, we review the use of SSRI-conjugated siRNA (small interfering RNA) to evoke faster and/or more effective antidepressant-like responses. Intranasal application of sertraline-conjugated siRNAs directed to 5-HT_{1A} receptors and SERT evoked much faster changes of pre- and postsynaptic antidepressant markers than those produced by fluoxetine.

Keywords: Ketamine, microRNA, prefrontal cortex, small interfering RNA, RNA interference

1. Glutamatergic drugs as fast-acting antidepressants?

1.1. Glutamatergic neurotransmission in mammalian brain

Part I of the present review analyses the neurobiological mechanisms involved in the limited efficacy and delayed onset of action of current antidepressant drugs, as well as the use of monoamine-based strategies to improve antidepressant action. In the second part, we review existing evidence supporting the use of glutamatergic drugs, with a particular emphasis on ketamine, as well as molecular strategies based on RNAi.

Glutamate is the most abundant neurotransmitter in mammalian brain. The cerebral cortex is made up of 80-85% projection pyramidal neurons that use glutamate as neurotransmitter plus 15-20% local circuit inhibitory interneurons that use GABA. In addition, a large number of subcortical brain areas, such as the thalamus, the hippocampus and the amygdala contain a very high proportion of glutamatergic excitatory neurons. Monoamine neurons represent a small fraction of brain neurons (e.g., there are ~250.000 serotonergic neurons in human brain, out of a total of 10^{11} neurons). Monoamines play essentially modulatory roles of glutamatergic and GABAergic signalling through their activation of G-protein-coupled receptors, with the exception of the 5-HT₃ receptor, the only ionotropic monoamine receptor.

Strong (ionic) actions of glutamate are mediated by three receptor subtypes, namely the AMPA, kainate and NMDA receptors. These receptors are ion channels, whose activation by glutamate allows extracellular Na⁺ and Ca²⁺ ions to enter and K⁺ ions to leave the neuron, thus evoking rapid and marked changes of the membrane potential, which subsequently allows for the generation of action potentials. In addition to these ionotropic receptors, glutamate can also act on a family of 8 subtypes of G-protein coupled metabotropic receptors, analogous to monoamine receptors, which are also suitable targets for drug development in various field of Psychiatry.

NMDA (or GluN) receptors are involved in a large number of key neurophysiological functions, such as long-term potentiation and synaptic plasticity, and play a role in several neurological and psychiatric disorders (Lau and Zukin, 2007). NMDA receptors are tetrameric ion channels composed of two NR1 and two NR2 (A, B, C, D) subunits (now termed as GluN1 and

GluN2A-D). A third type of NMDA receptor subunit (NR3, A and B; GluN3A-B) has been identified that changes the ionic sensitivity of the NMDA channel. The NMDA receptor ion channel is voltage-sensitive, i.e., the channel is blocked by Mg^{2+} ions when resting. Thus, in general, AMPA-induced depolarization is required for the release of Mg^{2+} ions and the subsequent activation of NMDA receptors.

The NMDA receptor contains several binding sites, including the site for glutamate and competitive antagonists, such as AP5 (or AP-V). Likewise, it contains several regulatory sites, such as the glycine site, outside the channel, and the Mg^{2+} and non-competitive antagonist sites (also called phencyclidine or PCP site), inside the channel. The dissociative anaesthetics ketamine and phencyclidine are non-competitive NMDA receptor antagonists. Due to their ability to evoke positive and negative symptoms of schizophrenia in healthy individuals and to aggravate them in schizophrenic patients, these agents have been used as a pharmacological model of schizophrenia (Krystal et al., 2003). The administration of these agents to laboratory animals evokes behavioural alterations (hyperactivity, abnormal startle response, etc.) that are reversed by antipsychotic drugs, suggesting their link with schizophrenia symptoms. In addition, evidence accumulated during the last decade or so indicates that psychotomimetic effects of ketamine are followed by a rapid, robust and persistent antidepressant effect.

1.2. Antidepressant effect of ketamine. Clinical studies

A first placebo-controlled, crossover study (Berman et al., 2000) showed that the i.v. infusion of a sub-anaesthetic dose of ketamine (0.5 mg/kg over 40 min) to 9 patients with unipolar or bipolar depression, resistant to standard treatments, evoked a robust and persistent antidepressant effect after the disappearance of the initial psychotomimetic phase. Maximal antidepressant effects were observed 72 h after ketamine infusion. A subsequent placebo-controlled, double-blind, crossover clinical trial, showed that ketamine infusion evoked an antidepressant response in 71% of treatment-resistant depressive (TRD) unipolar and bipolar patients. Significant improvement occurred as soon as 110 min after the beginning of ketamine infusion and remained for up to 7 days, with maximal antidepressant effects occurring after 24 h of infusion (Zarate et al., 2006).

Additional placebo-controlled studies confirmed these early observations in treatment-resistant unipolar and bipolar patients (reviewed in Monteggia and Zarate, 2015). For example, a

two-site, parallel arm, randomized controlled trial of a single infusion of ketamine was compared to an active placebo control condition, the anaesthetic midazolam, showing that response at 24 h was greater with ketamine than with midazolam (Murrough et al., 2013). Moreover, a double-blind, placebo-controlled trial showed that 27% of the patients treated with a single i.v. dose of ketamine (0.5 mg/kg) kept their response 4 wk post-treatment. Interestingly, an open-label study reported a very robust reduction of the severity in 14 emergency depressed patients with marked suicidal ideation using a lower ketamine dose (0.2 mg/kg, i.v.), yet at a much faster infusion rate (infusion in 1-2 min) (Larkin and Beautrais., 2011). Although these observations have not yet been replicated in a controlled way, they possibly indicate that the kinetics of ketamine and metabolites may be an important factor for clinical response.

Among responders to a single ketamine infusion there is substantial variability in time to relapse. Several studies have focused on the safety and efficacy of repeated-dose intravenous ketamine in TRD patients (Aan het Rot et al, 2010). Interestingly, an open label study in ~~unmedicated~~ TRD patients under washout of antidepressant medication, showed that, among responders, after up to six i.v. infusions of ketamine (3 times weekly over a 12 day period), the median time to relapse was 18 days. Remarkably, the response at 4 hr was highly predictive of a sustained response to subsequent infusions, although further studies are needed to test relapse prevention strategies after ketamine (see Monteggia and Zarate 2015 for review).

Given the widespread use of ketamine for pain treatment and the many administration routes employed, some have been explored for antidepressant treatments (for review see Abdallah et al., 2016, and Schoevers et al., 2016). Hence, sublingual and subcutaneous routes have been successfully used. Likewise, intranasal ketamine administration (single dose of 50 mg) evoked a significant improvement in depressive symptoms 24 hr after ketamine, compared to placebo. Among the large number of ongoing clinical trials examining antidepressant effect of ketamine (<https://clinicaltrials.gov/ct2/results?term=antidepressant+ketamine&Search=Search>), four of them use the intranasal route. Basically, NCT01304147 trial will evaluate the safety and efficacy of a single dose (50 mg) of intranasal ketamine in TRD, NCT03149991 trial will evaluate the acute efficacy of brexpiprazole plus intranasal ketamine added to ongoing antidepressant therapy in TRD and NCT02183272 trial will evaluate emergency intranasal ketamine (0.2 mg/kg) for treatment of suicidal ideation and NCT02644629 trial will evaluate intranasal vs intravenous administration of ketamine as an add-on to antidepressant therapy.

Ketamine is a racemic mixture of two enantiomers, R-ketamine and S-ketamine. The racemate has been used in most clinical trials and, to our knowledge, there is no information indicating which isomer is most involved in antidepressant effects. S-Ketamine is 4 times more potent than R-ketamine to bind to the PCP-site in membrane preparations and to reduce pain and sensory perception (Vollenweider et al., 1997). S-ketamine is better tolerated than the racemic mixture and has less psychotomimetic effects at an equianalgesic dose (0.5 and 0.25 mg/kg for ketamine and S-ketamine, respectively) (Paul et al., 2009). Likewise, S-ketamine is less potent than the racemic mixture to evoke pro-psychotic effects in mice and one study reported that R-ketamine was more potent and long-lasting than S-ketamine to evoke antidepressant effect in a mouse model of depression (Zhang, et al., 2014). However, it is still unknown whether these pharmacological and behavioural differences translate into clinical differences.

Other NMDA receptor antagonists have been tested as antidepressants in TRD. Memantine, a low-affinity noncompetitive NMDA receptor antagonist, did not induce a rapid antidepressant response (Ferguson et al., and Shingleton 2007). However, Lanicemine (AZD6765), also a low-affinity NMDA receptor antagonist, showed a comparable onset of antidepressant action, yet a lower response rate than ketamine at 80 min post-administration. The lack of psychotomimetic action by this agent suggests that antidepressant effects are not associated to the psychotomimetic action (reviewed in Monteggia and Zarate, 2015; Zarate et al., 2013).

As with conventional antidepressants, ketamine administration does not improve depressive symptoms in all patients. Thus, several studies focus on the use of potential biomarkers of ketamine antidepressant response in TRD (see Abdallah et al., 2016 for review). Interestingly, pre-treatment serine plasma concentrations are significantly lower in ketamine responder patients than in non-responders (Moaddel et al., 2014). Moreover, serum vitamin B12 concentration was significantly higher in bipolar patients responding to ketamine but no differences were found in the levels of homocysteine, a NMDA receptor agonist, despite the role of vitamin B12 in its metabolism. Likewise, magnetoencephalography revealed an increased activity in the anterior cingulate cortex in response to the presentation of affectively-charged stimuli predicted antidepressant response to ketamine (Salvadore et al., 2009).

Several studies indicate that brain-derived neurotrophic factor (BDNF) signalling is a key element in antidepressant action (Bjorkholm and Monteggia 2016; see below). In agreement, ketamine-induced antidepressant effect is dependent on increased BDNF signalling and ketamine

significantly increased plasma BDNF levels in responder patients 240 min after infusion. The reader is referred to two recent reviews on mechanisms underlying ketamine's antidepressant effect and BDNF signalling (Abdallah et al., 2016, and Bjorkholm and Monteggia 2016). Genetic studies show that MDD patients with the Val/Val BDNF allele at rs6265 are more likely to exhibit enhanced antidepressant response to ketamine than Met carriers. Interestingly, only patients responding to ketamine exhibited a simultaneous elevation of plasma BDNF levels and of the early slow wave activity (SWA; a biomarker of synaptic plasticity) suggesting that this effect may be associated the rapid antidepressant effects of ketamine. Also, pre-treatment metabolomics screening has been pointed to as predictor of response to ketamine treatment (Villaseñor et al., 2014).

Overall, these clinical observations indicate a very robust (in patients resistant to conventional treatments), rapid (in hours) and persistent (up to 10 or more days) antidepressant effects of ketamine after a single i.v. administration, despite that ketamine half-life is 2-3 h (Mion and Villeveille, 2013). Interestingly, the clinical antidepressant action of ketamine arises without severe side effects, restricted to its psychotomimetic activity, occurring during its i.v. infusion (Aan het Rot et al., 2010).

1.3. Mechanism of ketamine's antidepressant action

NMDA receptors have been involved for long in antidepressant effects in rodents (reviewed in Skolnick et al., 2009). However, the interest of the scientific and medical community on ketamine and NMDA receptors as potential target for antidepressant action raised only after the publication of the study by Zarate et al., 2006, which confirmed the initial results by Berman et al., 2000 (Fig. 1). A critical issue when attempting to understand the neurobiological basis of the antidepressant effect of ketamine is how this action can be differentiated from its psychotomimetic effects, since both occur at the same dose and administration route (typically 0.5 mg/kg i.v. infusion for 40 min). Time appears to be a critical variable for such a discrimination, since antidepressant effects 1) emerge after the finalization of the psychotomimetic effects (~1 h), which follow the rapid pharmacokinetics of ketamine (Mion and Villeveille, 2013), and 2) last for longer than psychotomimetic effects. Notwithstanding these temporal differences in the mode of action, a large number of experimental studies in humans and laboratory animals report on ketamine effects at different doses and times after administration, resulting in a wide range of concentrations of

ketamine and its metabolites. Overall, this makes difficult to provide a comprehensive view of the likely different mechanisms involved in psychotomimetic and antidepressant actions of ketamine.

Neuroimaging studies have provided information on brain areas sensitive to the action of ketamine, most likely related to its psychotomimetic effect. Hence, ketamine infusion to healthy individuals induced a focal activation of prefrontal and cingulate areas in parallel with the emergence of psychotic symptoms (Vollenweider and Kometer 2010). Likewise, ketamine increased glucose utilisation in the thalamus, an action shared by other hallucinogens, like psilocybin (Vollenweider and Kometer, 2010). Interestingly, S-ketamine, at psychotomimetic doses, increased cerebral metabolic rates of glucose in the prefrontal cortex (PFC) and the thalamus whereas R-ketamine, at equimolar doses and without psychotomimetic action, tends to decrease glucose utilisation across brain regions (Vollenweider et al., 1997). A recent study found that two ketamine doses (0.5 mg/kg and 0.2 mg/kg, i.v. infusion over 40 min) increased similarly glucose metabolism in PFC, an effect significantly correlated with the antidepressant response. Interestingly, PFC changes did not differ between patients with and without side effects and the patients treated with the lower dose reported similar side effects than the placebo group (Li et al., 2016). A functional magnetic resonance imaging (fMRI) study shows that ketamine decreased the resting state functional connectivity of the default mode network to the dorsal nexus, the pregenual anterior cingulate and the mPFC via its connectivity to the posterior cingulate cortex (Scheidegger et al., 2012). Some of these areas, and in particular those composing the default mode network, have been implicated in major depression. In particular, it has been reported an abnormally high contribution of the subgenual cingulate cortex and of the thalamus to the functional connectivity within the default mode network in depressed patients (Greicius et al., 2007). Likewise, the antidepressant response to ketamine has been negatively correlated with the pre-treatment functional connectivity of the pregenual anterior cingulate with the left amygdala during a working memory task (Salvadore et al., 2010).

A large number of preclinical studies have been carried out in search of the mechanisms of the antidepressant action of ketamine. Non-competitive NMDA receptor antagonists, including ketamine, memantine and MK-801 (dizocilpine), show antidepressant-like effects in animal models of depression such as the forced-swim test (FST) (Autry et al., 2011; Duman et al., 2016; Réus et al., 2010). These agents also increase monoamine release in PFC (Adams and Moghaddam, 2001; Amargós-Bosch et al., 2006). However, it is unclear whether such an effect can account for the

clinical antidepressant properties of ketamine, since ketamine-induced monoamine elevations are short-lasting and comparable to the effects of single administration of SSRI/SNRI. On the other hand, patients responding to ketamine had been previously treated with SSRI/SNRI for prolonged periods of time without success. The exact molecular/cellular and network mechanisms involved in ketamine's antidepressant action are still uncertain, although significant advances have been obtained in recent years.

1.3.1. Cellular actions of ketamine: effects on signalling pathways, BDNF and synaptogenesis

The increase in forebrain BDNF expression is essential for conventional antidepressant treatments and also for ketamine (Lepack et al., 2014; Abdallah et al., 2016; Bjorkholm and Monteggia 2016). BDNF binding to tropomyosin receptor kinase B (TrkB) regulates different intracellular pathways including the Akt-mTOR (protein kinase B/Akt- mammalian target of rapamycin) involved in synaptic plasticity mechanisms (Figure 2 in Abdallah et al., 2016 and Figure 2 in Duman et al., 2016) for potential pathways/mechanisms also putatively involved in the antidepressant action of ketamine).

Several initial targets have been suggested for ketamine, such as *i*) NMDA receptors on GABA interneurons (Homayoun and Moghaddam 2007; Quirk et al., 2009), *ii*) NMDA receptors on pyramidal neurons (Miller et al., 2016), and *iii*) AMPA-R on pyramidal neurons (Zanos et al., 2016) (see below). Beyond these initial targets, behavioural effects of ketamine are accompanied by changes in intracellular signalling mechanisms, likely leading to the reshaping of brain circuits responsible for clinical improvement. Hence, ketamine enhances neuronal plasticity and synaptogenesis, increasing the density and function of dendritic spines in the PFC (Li et al., 2010). It has also been shown that ketamine reverses the loss of dendritic spines in a rat chronic stress model. These effects were accompanied by an increased expression of synaptic proteins, such as glutamate A1 (GluA1) and Arc, after BDNF/TrkB-activation of Akt and mTOR signalling and inactivation of glycogen synthase kinase-3 beta (GSK3 β) (Duman et al., 2016). Interestingly, blockade of mTOR signalling prevented the effects of ketamine on hippocampal neurogenesis and its antidepressant-like effects in behavioural models (Autry et al., 2011), whereas the blockade of mGluR2/3 receptors induced mTOR-dependent rapid antidepressant-like effects (Dwyer et al., 2012). Moreover, it has been demonstrated that lithium (GSK3 inhibitor) combined with low subthreshold doses of ketamine potentiates its synaptogenic and antidepressant-like effects (Liu et al., 2013). On the

other hand, it has been reported that ketamine deactivates eEF2 (eukaryotic elongation factor downstream effector involved in translation) kinase (also called CaMKIII), reducing eEF2 phosphorylation and increasing BDNF expression. Likewise, inhibitors of eEF2 kinase such as rottlerin or 1-hexadecyl-2-methyl-3-(phenylmethyl)-1H-imidazolium iodide (NH125) induced similar behavioural responses than ketamine (Autry et al., 2011). Accordingly, antidepressant-like effects of ketamine were attenuated in inducible forebrain BDNF and conditional TrkB knockout mice (Autry et al., 2011) and, in eEF2 kinase knockout mice (Nosyreva et al., 2013). Moreover, it has been reported that ketamine induction of synaptogenesis and behavioural actions in the FST is impaired in mice carrying the BDNF Met/Met allele, thus confirming the importance of this pathway in the response to ketamine (Abdallah et al., 2016; Autry et al., 2011; Duman et al., 2016; Lepack et al., 2014). Remarkably, the infusion of BDNF-neutralizing antibodies into the mPFC prevented the behavioural actions of ketamine in the FST (Lepack et al., 2014) indicating this brain area as a target in ketamine's antidepressant actions (see below). Likewise, ketamine stimulated the phosphorylation and nuclear export of histone deacetylase 5 (HDAC5) attenuating its repressive influence on the transcription of myocyte enhancer factor 2 (MEF2) and leading to the upregulation of several genes as Arc (Arg3.1), Klf6 (Krueppel-like factor 6) and Egr1 (Early growth response protein 1) in the hippocampus. Accordingly, shRNA-induced HDAC5 knockdown in rat hippocampus blocked and occluded the behavioural actions of ketamine (Choi et al., 2015). The critical role of HDAC5 in the therapeutic mechanisms for antidepressant treatments has been previously demonstrated for classical antidepressant drugs (Tsankova et al., 2006).

1.3.2. Effect of ketamine on brain networks

As indicated above, ketamine, PCP and MK-801, are non-competitive NMDA receptor antagonists, interacting with the so-called PCP site, inside the NMDA receptor channel. This indicates that these agents can block the action of glutamate at NMDA receptors located in cellular compartments that have been previously depolarized, in order to release the Mg^{2+} ion that blocks the NMDA channel in resting conditions. Thus, ketamine, PCP and MK-801 likely block the excitatory actions of glutamate in more active neurons, which are depolarized for more prolonged periods of time, showing a functional cellular selectivity. Interestingly, the systemic administration of PCP evoked a marked and paradoxical overall increase of the activity of pyramidal neurons in rat PFC (Kargieman et al., 2007). In parallel, PCP increased the expression of the immediate early gene c-fos in cortical pyramidal

neurons and in subcortical glutamatergic neurons, in particular thalamic nuclei, where the neuronal discharge rate was also enhanced by PCP (Santana et al., 2011). Interestingly, PCP did not increase c-fos expression in thalamic reticular GABAergic neurons that provide a feed-forward inhibition to the rest of thalamic nuclei (Santana et al., 2011). Subsequent studies showed that PCP actually inhibits the activity of reticular GABA neurons, thus disinhibiting thalamocortical networks (Troyano-Rodriguez et al., 2014). Systemic ketamine administration preferentially inhibited fast-spiking interneurons in rat orbitofrontal cortex (Quirk et al., 2009). Overall, these observations suggest an in vivo cellular selectivity of PCP and ketamine for NMDA receptors located on GABA neurons, either cortical interneurons or projection neurons, such as those of the reticular thalamic nucleus. In line with these observations, Ro25-6981, a GluN2B-selective antagonist that elicit antidepressant-like activity in the FST (Jimenez-Sanchez et al., 2014), reduced synaptic NMDA responses in a subpopulation of GABAergic interneurons but not in pyramidal neurons in hippocampal slices (Hanson et al., 2013). However, other studies suggest a role of NMDAR subunit GluN2B on pyramidal neurons, since genetic deletion of NMDAR subunit GluN2B from forebrain pyramidal neurons mimics and occludes ketamine's actions on depressant-like behaviour and excitatory synaptic transmission (Miller et al., 2016).

Despite the common actions of non-competitive NMDA-R antagonists on GABA cells, it is unclear whether they affect brain circuits in a similar way. In common the above PCP studies in rats indicating an activation of thalamocortical circuits, PET-scan studies in healthy volunteers reported that ketamine increased glucose metabolism with a similar pattern to that observed in c-fos and electrophysiological studies with PCP, i.e., in prefrontal and cingulate cortices, thalamus, amygdala, etc. (Vollenweider and Kometer 2010). Furthermore, fMRI in rhesus monkeys showed that ketamine infusion induces a preferential activation of several brain areas such as the cingulate gyrus and the thalamus (Maltbie et al., 2016). However, unlike PCP and MK-801 (Kargieman et al., 2007; Vaisinen et al., 2004), subanesthetic ketamine administration did not increase c-fos expression in rat thalamus (Duncan et al., 1998). The reasons for these discrepancies are poorly known and may involve species differences (e.g., Vollenweider and Kometer, 2010) as well as a different selectivity of ketamine for NMDA-R in different cellular types at the various doses and routes used.

As mentioned above, in contrast to the above observations suggesting that ketamine and other NMDA-R antagonists disinhibit excitatory neurotransmission in the PFC and anatomically-related brain areas, it has been suggested that ketamine may be exerting its antidepressant action

through the direct inhibition of cortical pyramidal neurons (Miller et al., 2016). This view was based on the observation that the selective *in vivo* deletion of GluN2B from cortical pyramidal neurons mimicked ketamine's actions on behaviour (Miller et al., 2016). In agreement with these observations, ketamine evoked the same behavioural responses in wild-type mice and in mice lacking NMDA receptors in parvalbumin neurons (Pozzi et al., 2014).

Interestingly, ketamine increased the functional connectivity in the hippocampal-prefrontal pathway in rats and humans (Grimm et al., 2015) and a recent study revealed that ketamine reconfigures large-scale brain network connectivity similarly in rats and humans, leading to a more segregated information processing, a key information for the translational value of rodent studies (Becker et al., 2016).

1.3.3. Involvement of AMPA receptors in the antidepressant actions of ketamine

As also observed for other fast-acting antidepressant treatments (Jimenez-Sanchez et al., 2015; Duman et al., 2016), ketamine's action involves the activation of AMPA receptors, an effect that may result from the blockade of NMDA-R in subpopulations of GABA neurons, but also from the direct activation of AMPA-R by a ketamine metabolite, as summarized below.

Using [¹H]-magnetic resonance spectroscopy, it was found that ketamine increases glutamine –a marker of glutamatergic transmission- in the anterior cingulate of healthy individuals (Rowland et al., 2005). Likewise, using [¹³C]-NMR in rats, it was reported that ketamine increases cortical glutamate, glutamine and GABA labelling (Chowdhury et al., 2012), in agreement with the increased glutamate efflux previously reported with microdialysis (Adams and Moghaddam 2001). These observations suggest that ketamine enhances presynaptic glutamatergic neurotransmission.

Likewise, several studies show that AMPA receptor activation is critical for the antidepressant-like action of ketamine and other NMDA antagonists (Autry, 2011; Li, 2010 2017). Hence, the antidepressant effect of ketamine was blocked by microinjection into the mPFC of the AMPA receptor antagonist, NBQX (Fukumoto et al., 2016). This agent also blocked the antidepressant-like effect produced by the local application of dihydrokainic acid (DHK, an inhibitor of astrocytic glutamate uptake) and of S-AMPA in rat infralimbic cortex (Gasull-Camos et al., 2017). On the other hand, chronic AMPA treatment of rats evoked antidepressant-like effects in several

behavioural tests, and synergized with ketamine showing also increases in markers of hippocampal neurogenesis and synaptogenesis (Akinfiresoye and Tizabi, 2013).

The increased AMPA-R activation by ketamine may be explained by the increase in presynaptic glutamatergic function produced by non-competitive NMDA-R antagonists and the concurrent blockade of NMDA-R. However, given the short half-life of ketamine and its main metabolite, norketamine, this process cannot explain the emergence of antidepressant effects after the pro-psychotic actions of ketamine (i.e., when plasma and brain concentrations are maximal) nor their persistence for several days. A recent report has shed light into this temporal discrepancy, showing that, at least in mice, the metabolism of racemic ketamine to (2S,6S;2R,6R)-hydroxynorketamine (HNK) is required for antidepressant-like effects (Zanos et al., 2016). The same study found that the antidepressant-like actions of the (2R,6R)-HNK enantiomer are independent of NMDA-R blockade but involve early and sustained activations of AMPA-R and lacks ketamine-related side effects (Zanos et al., 2016) (Fig 2). Nevertheless, other teams have reported that R-ketamine shows greater potency and longer lasting antidepressant effects than its metabolite (2R,6R)-hydroxynorketamine (Yang et al., 2017).

1.3.4. Ventral anterior cingulate cortex: a regional target for ketamine?

Over the last two decades, the ventral anterior cingulate cortex (vACC) has emerged as a key area in the pathophysiology of MDD. Early neuroimaging studies reported a decreased energy metabolism in the subgenual cingulate gyrus of depressed patients (Drevets et al., 1997). Also, individuals with depression displayed reduced levels of immediate early genes as zif268 mRNA in the anterior cingulate cortex (Brodmann area 24) and this effect was even most apparent in subjects without medication at the time of death (Covington et al., 2010). However, other studies suggest an increased metabolism in vACC, including Brodmann area 25 (Cg25) (Mayberg et al., 2005). Also, in treatment-refractory MDD patients subjected to anterior cingulotomy, a high pre-operative metabolism rate in the left subgenual prefrontal cortex and left thalamus were associated with a better outcome (Dougherty et al., 2003). Likewise, an increased activity at rest in this area before treatment onset predicted better antidepressant responses (Mayberg et al., 1997) whereas a decreased activity after treatment has been associated to clinical response (Mayberg et al., 2005; Mayberg et al., 2000). Thus, metabolic activity in this area has been postulated as a predictor of

treatment response in MDD. Likewise, it has been suggested that antidepressant treatments should decrease the activity of the rostral anterior cingulate cortex (rACC) (Pizzagalli et al., 2011).

These differences between studies (increased vs decreased activity) may be related to the different area analyzed. In agreement, a recent study reported on a temporal and regional selectivity of ketamine effects on glutamatergic levels in two distinct subdivisions of ACC (pgACC and the anterior midcingulate cortex) associated with the time course of the antidepressant response (Li et al., 2017). Specifically, the ratio glutamine/glutamate was found to be increased in the pgACC 24 h after i.v. ketamine administration in healthy subjects. Remarkably, alterations of astroglial glutamate activity have been reported in the anterior cingulate of depression patients (Bernstein et al., 2015).

Early neuroimaging studies indicated that ketamine mostly increased energy metabolism in the PFC (see Matbie et al. 2017 for review). However, the situation appears to be more complex, with different effects on the various PFC subdivisions, probably related with the different connectivity of these areas with other brain areas and the reciprocal interaction between the vACC areas (limbic region) and the dorso-lateral prefrontal cortex (dLPFC, associated with cognitive functions) (Hoover and Vertes 2007).

Despite the obvious limitations in the comparison of primate and rodent brains, the infralimbic (IL) and prelimbic (PrL) subdivisions of the rodent mPFC are considered, respectively, the homolog areas of the vACC and dLPFC in the humans (Hoover and Vertes 2007). These areas appear to exert a mutual inhibitory influence, since the optogenetic activation of IL pyramidal neurons inhibited PrL pyramidal neurons (Ji and Neugebauer 2012). Interestingly, the optogenetic stimulation of the ventral mPFC in mice elicited a potent antidepressant-like effect (Covington et al., 2010). An important step toward the identification of the mechanisms underlying the antidepressant actions of ketamine came from a recent study in rats showing how optogenetic stimulation of the IL reproduces ketamine's antidepressant actions (Fuchikami et al., 2015) in association with an increase in the number and function of spines and synapses, respectively, of layer V pyramidal neurons. Additionally, neuronal inactivation of the IL by muscimol infusion (a GABA_A-R agonist), blocked the antidepressant effect of systemic ketamine whereas ketamine microinfusion into the IL reproduced the behavioural actions of systemic ketamine administration (Fuchikami et al., 2015). These evidences suggest that ketamine's antidepressant effects are mediated by an increase of the IL activity. Given the connectivity between the IL and midbrain

serotonin raphe neurons, these antidepressant-like effects would be associated also by an augmentation of serotonergic neurotransmission. Accordingly, deep brain stimulation of the IL but not PrL induced antidepressant effects in rats (Jimenez-Sanchez et al., 2015 2016). A similar regional difference has also been for the effects of the astroglial uptake inhibitor DHK and for S-AMPA (Gasull-Camós et al., 2017). Interestingly, the antidepressant-like effects of AMPA-R stimulation in IL appear to be mediated by an increased serotonergic neurotransmission, which points to the IL-raphé pathway as a crucial network in MDD treatment (Gasull-Camós et al., 2017).

Likewise, the involvement of serotonergic transmission in ketamine's antidepressant action has been revealed in FST showing that 5-HT depletion with *p*CPA (irreversible 5-HT synthesis inhibitor), attenuated the antidepressant effect of local (in mPFC) or systemic ketamine administration (Fukumoto et al., 2016). Also, antidepressant-like effects of deep brain stimulation (DBS) were prevented by 5-HT depletion in rats (Hamani et al., 2010). These observations highlight the crucial role played by the PFC-raphé pathway in mood control and stress resilience. As reviewed in part I, raphe 5-HT neurons are extremely sensitive to physiological and pharmacological changes in mPFC. Interestingly, the optogenetic stimulation of mPFC neurons projecting to DR induced a rapid and reversible effect on active escape behaviour that was not observed after the non-selective stimulation of mPFC pyramidal neurons nor after stimulation of DR 5-HT neurons (Warden et al., 2012). As mentioned, ketamine alters the functional connectivity between several brain areas. It has been reported that ketamine increases PFC metabolic activity while reducing metabolic activity in the dorsal reticular thalamic nucleus (Dawson et al., 2013) and alters the functional connectivity of 5-HT, NA and Ach systems. Moreover, ketamine enhanced 5-HT or hypocretin-induced excitatory postsynaptic currents (EPSC) responses in apical dendritic spines of layer V pyramidal neurons (Li et al., 2011) suggesting a reinforcement on excitatory thalamocortical connectivity. Likewise, frequency and amplitude of EPSC in mPFC layer V pyramidal neurons induced by 5-HT or hypocretin were decreased by chronic unpredictable stress and these deficits were rapidly reversed by ketamine (Li et al., 2011). Finally, ketamine has been reported to increase apical EPSC responses in all mPFC subregions (anterior cingulate, PrL and IL) but selectively reinforces corticotropin releasing factor (CRF)-activated amygdala inputs to basal dendrites in layer V pyramidal cells in the anterior cingulate and PrL but not IL pointing to a selective action of ketamine on apical versus basal amygdala inputs in this area (Liu et al., 2015).

2. RNAi as a potential target for antidepressant treatments

MDD is a complex disorder resulting from the interaction of neurobiological, genetic, cultural, and environmental factors. There is increasing evidence that MDD is associated with altered structural and synaptic plasticity, stress-related pathology, and to a certain extent, genetic polymorphism (Peña et al., 2014; Ota and Duman, 2013). Disruptions across cellular networks and aberrant information processing in mood- and cognition-regulating circuits may alter synaptic and structural plasticity, leading in the end to the emergence of MDD symptoms. Research over the last decade has enabled to identify epigenetic mechanisms as important effectors to regulate programs of gene expression associated with depression-related behaviors, antidepressant action, and resilience to depression in animal models, with a high translational value (Sun et al., 2013). Epigenetic mechanisms include histone and DNA modifications such as acetylation, ubiquitination, sumoylation and methylation in individual neurons (Nestler et al., 2015). Understanding the epigenetic regulatory mechanisms in MDD and their therapeutic applications is an important avenue for future investigation.

More recently, small non-coding endogenous RNAs (ncRNAs), microRNAs (miRNAs), have emerged as post-transcriptional regulators of gene expression, although not epigenetic *per se*. miRNAs play a role in almost all biological processes, such as cell proliferation, development, differentiation, and programmed cell death. More than 1400 miRNA genes have been already identified, each of which may target a large number of different messenger RNAs (mRNAs), which illustrates their crucial role as determinants of cellular states and fates (Lin and Gregory, 2015). miRNAs may impair the translation of their target mRNA or promote its degradation (see below RNA interference), but they can also act as translation activators or even impair transcription by binding to gene promoters (Krol et al., 2010). Given the cellular and transcriptional complexity of the nervous system, it is not surprising that ~75% of annotated miRNAs are expressed in the brain (Kosik, 2006).

Early studies reported on miRNA expression in broad brain areas. However, the use of new technologies allowed to study miRNAs brain distribution at both cellular and sub-cellular levels. For instance, using combining immunoprecipitation of tagged in transgenic Ago2 mice with cell type specific Cre/Lox system, it has been possible to identify profiles of miRNA:mRNA complexes in different subtypes of GABAergic interneurons and excitatory pyramidal cells from neocortex (He et

al., 2012). Likewise, primary miRNA gene transcripts are processed within the nucleus but also in dendrites, highly enriched near synapses (reviewed in Smalheiser, 2014).

Brain miRNAs contribute to both brain development and neural function, including neurogenesis, neuronal differentiation, and synaptogenesis, thus offering many opportunities to finely tune the functional properties of neuronal subpopulations (Mercer et al., 2008; Sun and Shi, 2015). Moreover, miRNAs play critical roles in cell fate determination of neurons and glia, by suppressing specific target genes. Thus, miR-124 and miR-29 are among the best-studied miRNAs and regulate the differentiation of progenitors into neuronal or glial pathways. Whereas miR-124 and miR-128 are primarily expressed in neurons; miR-23, miR-26 and miR-29 occur in large amounts in astrocytes (Makeyev et al., 2007; Sun et al., 2015). The dysregulation of miR-124 has been related to neurodegeneration, neuroimmune disorders and CNS stress among others (Sun et al., 2015). It was previously indicated to be a key regulator of adult neurogenesis and one of its targets is CREB (Cheng et al., 2009). On the other hand, in the olfactory neuroepithelium, the loss of the enzymatic Dicer complex (Fig. 3; see also next section) has been associated with a reduced neuronal progenitor cell population and lowered rate of neuronal progenitor maturation (Choi et al., 2008). Some miRNAs are also implicated in neurological functions such as learning and memory. Hence, miR-132, miR-134 and miR-let-7 are suggested to play a crucial role in the formation and plasticity of synapses, and miR-124a and miR-125b have been associated to axonal outgrowth (Vo et al., 2005; Schratt et al., 2006). Currently available data indicate that miRNAs may be important cellular regulators contributing to normal and pathological brain function (Alural et al 2016). Likewise, changes in miRNA expression and function are involved in the pathophysiology of MDD as well as in antidepressant actions. Given the critical need for new diagnostic markers and disease-modifying treatments, expanding the focus of genomic studies of neuropsychiatric disorders such as MDD to include the role of ncRNAs (miRNA and small interfering RNA – siRNA) is of growing interest.

2.1. RNA interference (RNAi)

RNAi is a method to selectively and efficiently knockdown the expression of a targeted mRNA. RNAi is also an endogenous process that regulates several cellular functions. Specifically RNAi is reported to function in the cellular defense against viral infections, in controlling the expression and mobility of transposons, which are mobile genetic elements, and in the regulation of endogenous gene

expression (Hannon, 2002). The interested reader is referred to review articles on basic aspects of the biochemistry of RNAi (Meister and Tuschl, 2004). Briefly, RNAi refers to the process by which double-stranded RNA (dsRNA) molecules silence a target gene through the specific degrading of its mRNA or blocking its translation (Fig. 3). Both molecules, miRNA and siRNA, are derived from long dsRNA. This precursor is processed by a specific RNase-III family of enzymes termed Dicer, which generates short fragments, usually 21–23 nucleotides (nt) in length. In addition to the differences in biogenesis, the siRNA duplex is a strict duplex of Watson-Crick base pairing; conversely, the miRNA is often an imperfect duplex with mismatches or bulges (Tang, 2005). To be functional, short RNAs need to be incorporated into a protein complex known as the RNA- induced silencing complex (RISC). The key protein component of this effector complex is the Argonaute (AGO) protein, member of an evolutionally conserved protein super family. After RISC assembly and maturation, specific gene transcripts or mRNAs, are recognized and bound by the short RNAs to RISCs, based on sequence complementarities, leading to site-specific cleavage or translational repression of the mRNAs. Thus, cellular RNAi pathways can be manipulated by introducing exogenous siRNAs or by transfecting DNA-vector encoding shRNAs (small hairpin RNAs) as well as vector-based miRNAs (Paddison et al., 2002; Stegmeier et al., 2005).

Because of its potential to facilitate understanding of the roles of ncRNAs in the pathophysiology of disease and to develop new treatment strategies, the discovery of RNAi caused a great deal of excitement in the biomedical world. The first applications in mammals, however, were highly disappointing due to the interferon response induced by the introduction of dsRNAs into cells. Fortunately, this obstacle was overcome by the identification of immunostimulatory motifs that allowed the design of siRNAs or shRNA that can mediate RNAi with minimal immune activation (Judge et al., 2005; Robbins et al., 2008). Ten years ago, a review appeared in *Molecular Therapy* that addressed the rapid ascent of RNAi from discovery to a routine tool used in research with therapeutic aspirations (Behlke et al., 2006). One of the concluding statements of the review was as follows: “RNAi based drugs are already in clinical trials and it is hopeful that a siRNA therapeutic will receive US Food and Drug Administration approval in the not so distant future.” At that time, around 100 published reports on the *in vivo* use of RNAi could be found. Today, a search in PubMed with the specified keywords “RNAi/siRNA *in vivo*” retrieves almost 9000 references, 1000 of them related with brain disorders. The next sections contain little discussion about basic aspects of RNAi biochemistry and focus on recent advances on the use of miRNAs and siRNAs as an *in vivo* research tools or new therapeutic approaches for MDD.

2.1.1. Role of microRNAs in depression and antidepressant treatments

Accumulating evidence supports the association between several psychiatric disorders, including MDD, and changes in miRNA regulation or function. Such changes were identified in postmortem brain tissue samples, cerebrospinal fluid (CSF) or peripheral blood of MDD patients. Alterations in miRNA activity can occur at different levels: 1) the machinery genes related to miRNA biogenesis, 2) the miRNA promoter sequences, 3) the sequences responsible for pre-miRNA cleavage, 4) the miRNA binding sites in target genes, and 5) the seed sequences (Hogg and Harries, 2014). Here we summarize preclinical and clinical studies showing an altered miRNA expression in MDD (Table 1) as well as the involvement of miRNAs in antidepressant actions (Table 2).

Interestingly, predicted targets of altered miRNAs play significant roles in a wide variety of biological effects, including cell growth, differentiation, axon guidance and extension, synaptic transmission, learning and memory, which have been associated to the pathophysiology of MDD. Hence, Lopez et al., (2014) reported downregulation of miR-1202 in depressed individuals. miR-1202 targets the expression of the gene encoding metabotropic glutamate receptor-4 (GRM4), which was upregulated in MDD patient samples. In addition, different studies have identified altered miRNA levels in the blood and CSF of MDD patients, which were suggested as potential biomarkers of MDD (Table 1). Thus, miR-144-5 targets the protein C (PKC) and Wnt/ β -catenin pathways associated with depression (Wang et al., 2015), and miR-144-5 is involved in the response to mood stabilizer treatment and stress responses (Zhou et al., 2009). Others downregulated miRNAs: miR-30a-5p, miR-34a-5p and miR-221 were identified in blood of MDD patients. These miRNAs target different genes such as serine protein kinase (AKT), serotonin receptors (HTR2C), corticotropin-releasing hormone receptor (CRHR1) and glutamate transporters (SCL1A2) as well as several pathways related to neuronal brain function of the depression (Wan et al., 2015).

Regarding a possible involvement of miRNAs as downstream effectors in the action of antidepressant drugs, effects of different drugs on miRNA expression have been reported and are summarized in the Table 2. Among them, some cell line and animal studies showed that miR-16 is involved in brain development and function. It is also involved in MDD and is affected by antidepressant treatments (Tables 1 and 2). Bai et al. (2012) showed that maternal deprivation in rats increased miR-16 expression in the hippocampus, an effect associated to depressive-like

behavior (Table 1). Conversely, fluoxetine decreased miR-16 in mouse hippocampus and its neutralization further exerted an antidepressant-like effect in behavior tests (Launay et al., 2011, Table 2). miR-16 was identified as a miRNA with complementarity to the 3'-untranslated region of the mRNA of serotonin transporter (SERT) gene, which is a key regulatory protein of serotonergic transmission, associated to MDD (Baudry et al., 2010, Table 2) and the target for most antidepressant drugs. Baudry et al. (2010) first reported that miR-16 was found at higher levels in noradrenergic than in serotonergic cells, its reduction in noradrenergic neurons causing de novo SERT expression. However, a clinical study with 36 MDD patients and 30 healthy controls showed that miR-16 in cerebrospinal fluid (CSF) of MDD patients –but not in blood- was significantly lower than in healthy subjects (Song et al., 2015, Table 1). Overall, these observations suggest that CSF miR-16 deserves further examination in new studies as a potential biomarker of MDD.

More recently, miR-135 was described as a specific miRNA “fingerprint” of 5-HT neurons, regulating both SERT and 5-HT_{1A} receptor transcripts (Issler et al., 2014), two of the main players in antidepressant drug action. Intriguingly, miR-135 levels were upregulated after administration of several antidepressant drugs. Likewise, miR-206 has been identified as a critical miRNA for modulating the BDNF expression induced by ketamine. The overexpression of miR-206 in cultured neuronal cells using pre-miR-206 attenuated ketamine-induced up-regulation of BDNF levels (Yang et al., 2014). Therefore, given the role of miRNA as potential regulators of neurogenesis and neural plasticity, and the studies suggesting that miRNA processing may contribute to depression risk and treatment response, it is anticipated that miRNAs may become new diagnostic and treatment tools in the field of MDD.

2.1.2. Preclinical development of siRNA-based antidepressant therapies

siRNAs are synthetic mediators of RNAi, dsRNA molecules of 21 to 23 base pairs (bp) in length, specifically designed to silence the expression of target genes. They can be introduced exogenously into the cell or organism in short (21–23 bp) form or in the form of long dsRNA molecules. These dsRNAs are processed by endogenous RNAi machinery after introduction into the cell (Fig. 3). First, the cytosolic enzyme Dicer cleaves long dsRNAs into shorter fragments (siRNAs), leaving two nucleotide (2-nt) 3' overhangs and 5' phosphate groups (Meister et al., 2004). siRNAs are recognized by the AGO2–RISC enzyme complex, where one of the strands is degraded and the other (mostly

antisense) strand is left as a guide to find target mRNA sequences. Unlike miRNAs, siRNAs bind sequences with perfect or nearly perfect complementarity and cause cleavage of targets instead of translational suppression. Because they can efficiently silence target gene expression in a sequence-specific manner, siRNAs became necessary tools to study single gene function both *in vitro* and *in vivo* and are an attractive new class of therapeutics, especially against undruggable targets for the treatment of brain disorders and other diseases (Ozcan et al., 2015).

2.1.3. Considerations for the *in vivo* use of siRNAs

Despite the many advantages of siRNAs to treat brain diseases, many challenges remain, including: 1) off-target effects, 2) rapid degradation, 3) immune response and, 4) poor cellular uptake and *in vivo* delivery, and 5) cellular selectivity. Rational design strategies, predictive models based on second-generation algorithms, chemical modifications and nanocarriers offer significant opportunities to overcome some of the above problems.

Off-target effects. Although siRNAs are designed to knockdown specific targets, they may also silence an unknown number of unintended genes. Two factors may account for off-target effects. On the one hand, siRNAs can tolerate several mismatches at the target mRNA and retain their ability to silence those targets with imperfect complementarity (Snove et al., 2004). On the other hand, siRNAs may promiscuously enter endogenous miRNA pathways (Doench et al., 2003). miRNAs recognize targets with perfect complementarity to their 'seed regions' composed of nucleotides 2–8. Complementarity of remaining nucleotides is less important for recognition. Because siRNAs are nearly identical to the related class of miRNAs, they can recognize mRNAs with their seed region and then lead to degradation of an unpredictable number of **mRNAs**. Many efforts are being made to limit off-target risk without reducing siRNAs efficacy, including chemical modifications in the sequences and variations in siRNA design.

Regarding siRNA efficacy, early reports showed that not all siRNAs are equipotent. It was reported that different siRNAs targeting different parts of the same mRNA sequence have varying RNAi efficacies, and only a limited fraction of siRNAs has been shown to be functional in mammalian cells. Among randomly selected siRNAs, 58–78% were observed to induce silencing with greater than 50% efficiency and only 11–18% induced 90–95% silencing (Chalk et al., 2004). Most of the first-generation design tools were developed from a relatively small number of validated siRNAs and

focused on specific features of the siRNA itself (overall guanine–cytosine content, asymmetric thermostability, etc.) with little or no consideration given to the structure of the target mRNA. More recently, second-generation algorithms have been developed taking into account the structure in the target mRNA, which have significant effects on the ultimate potency of a site to mediate RNAi silencing (Ozcan et al., 2015; Rettig and Behlke, 2012).

Furthermore, it is well established that chemically synthesized siRNAs have a host of issues that affect functionality, especially when used in an *in vivo* setting where the full repertoire of immune competent cells are present. RNAi is a mechanism involved in the innate immune response to protect cells from invasion by nucleic acids of pathogens such as viruses and bacteria. Several studies revealed that siRNAs themselves can activate innate immunity by inducing interferon expression, even at low concentrations (Judge et al., 2005; Robbins et al., 2008). Protein kinase R (PKR) and toll-like receptor (TLR) 3 signaling pathways may be involved in sequence-independent immune activation by siRNAs. In addition, several siRNAs stimulate production of proinflammatory cytokines via TLR7 and TLR8 in a sequence-dependent manner. Some sequence motifs such as 5'-UGUGU-3' (Judge et al., 2005) or 5'-GUCCUCAA-3' (Hornung et al., 2005), some secondary structures as well as the uridine content of the sequence, are important factors for immune activation by these pathways. Chemical modifications such as 2'-O-methylation were shown to prevent immune activation by siRNAs.

In addition, nuclease stability, half-life, biodistribution and delivery are all factors to consider for improvement in making siRNAs suitable for any *in vivo* use, especially therapeutics. Several reviews provide thorough discussions of the chemical modification strategies available for use in siRNAs (Behlke, 2008; Gagliardi and Messere, 2010). Thus, together with rapid excretion kinetics, the susceptibility to degradation by nucleases is a major problem leading to short half-life (15 min to 1 h) in plasma, an important limitation for the use of siRNAs. However, some chemical modifications have been shown to protect siRNAs from nuclease degradation without interfering with siRNA silencing efficiency (Behlke, 2008), and some others, such as phosphorothioate modification or hydrophobic ligands (e.g., cholesterol), were shown to increase protein binding and extend serum lifetime (Soutschek et al., 2004; Ozpolat et al., 2010). Besides these modifications, nanocarriers are important tools providing protection against both rapid renal excretion and nuclease cleavage during delivery of siRNAs to target tissues (Ozpolat et al., 2010). Furthermore, another important aspect involves the delivery of siRNAs to target selected tissues/cells as well as

the entrance of siRNAs to their sites of action in the cytosol or nucleus (Juliano et al., 2008). Several strategies have been developed to deliver oligonucleotides in a selective manner using cationic lipid, polymer complex or antibody-mediated endocytosis (Song et al., 2005; Vornlocher, 2006). Likewise, another strategy is to use receptor/transporter-mediated targeting for pharmacologically effective delivery of siRNAs to specific cells, in which a ligand that selectively binds to a cell surface receptor/transporter is chemically conjugated to the oligonucleotide (Juliano et al., 2008; Bortolozzi et al., 2012; Ferrés-Coy et al., 2016). Several examples will be discussed in the next section showing the potential advantage of this strategy to selectively accumulate the siRNA into some neuronal types.

2.2. siRNAs and monoaminergic pathways

Several preclinical studies in rodent models have also indicated the feasibility to target brain genes in monoaminergic neurons involved in mood and cognitive disorders using exogenous siRNA molecules, independent of their perceived druggability (Shishkina et al., 2004; Thakker et al., 2004, 2005; Bortolozzi et al., 2012; Ferrés-Coy et al., 2013a,b; 2016). Some of these genes encoding for the monoamine transporters are key regulators of dopaminergic, noradrenergic and serotonergic neurotransmission as they control the active fraction of these monoamines. These transporters are also implicated in the pathophysiology and pharmacotherapy of neuropsychiatric disorders such as attention-deficit hyperactivity disorder, drug dependence, Parkinson's disease and mood disorders, including anxiety and MDD. As outlined above, SSRI and SNRI are first choice treatments for MDD and other psychiatric disorders but show important limitations in terms of efficacy and speed of action (Trivedi et al., 2006). Given the key role of transporters controlling brain monoamine transmission, Thakker et al., (2004, 2005) pioneered the field by silencing the DA and 5-HT transporters (DAT and SERT, respectively) using *in vivo* RNAi. In these studies, the administration of unmodified siRNAs targeted DAT or SERT into the dorsal third ventricle of mice during two weeks specifically reduced the mRNA levels of the DAT and SERT by ~30%. This reduction was similar to that previously observed in an early study using plasmids containing a partial antisense sequence against the serotonin transporter gene (28% reduction observed 7 days after intra-raphe plasmid infusion) (Fabre et al., 2000). More recently, we showed that the infusion of small amounts of an unmodified siRNA targeting SERT into the mouse dorsal raphe nucleus during four days can efficiently and selectively reduced SERT mRNA and protein expression by ~37% (Ferrés-Coy et al.

2013a). RNAi-induced reduction of SERT level triggers remarkable effects on serotonergic function, faster and more efficient than those produced by the pharmacological SERT blockade induced by SSRI fluoxetine (Ferrés-Coy et al., 2013a).

However, the utility of RNAi-based silencing strategies for MDD treatment is severely compromised by the extreme difficulty to deliver oligonucleotide sequences to their neuronal functional sites, due to the need to cross several biological barriers after administration, as mentioned before (Juliano et al., 2008). In order to overcome this problem, we designed and developed a siRNA sequence targeting SERT and other genes expressed by serotonergic neurons conjugated with the SSRI sertraline -which selectively binds to SERT- to successfully transport and internalize the siRNA molecule into 5-HT neurons (Bortolozzi et al., 2012; Ferrés-Coy et al., 2016; Ferrés-Coy et al. personal communication). The working hypothesis was that the presence of the SSRI would allow the selective enrichment of the conjugated siRNA in 5-HT neurons, through the dense network of 5-HT axons in rodent brain (ca. 10^6 axonal varicosities/mm³ in rat cortex), where the targeted transporter (SERT) is differentially expressed. Interestingly, the labeling of a sertraline-conjugated siRNA with Alexa 488 enabled its identification in the midbrain raphe, supporting its selective accumulation by 5-HT neurons (Ferrés-Coy et al., 2016).

Using this approach, we showed that 7 days of intranasal administration of the conjugated siRNA targeting SERT elicited very fast and robust antidepressant-like responses in control and corticosterone-treated mice, comparable than those evoked by a 28-day fluoxetine (10 mg/kg·day) treatment (Ferrés-coy et al. 2016). These responses included: *i*) reduced 5-HT_{1A} autoreceptor expression/function, *ii*) facilitated forebrain 5-HT neurotransmission, *iii*) increased proliferation of neuronal precursors, *iv*) increased expression of growth factors (e.g., BDNF, VEGF) and genes promoting neurite outgrowth (for example, neuritin, PSD95) and *v*) increased hippocampal dendritic complexity and synaptic plasticity. All these variables are predictive of clinical antidepressant action. In addition, short-term (7-day) conjugated SERT siRNA treatment normalized stress-induced depressive-like behaviors, which were only sensitive to 28-day fluoxetine treatment. Interestingly, the above actions were produced by the intranasal administration of very small doses (2.1 nmol/day) of conjugated siRNA, illustrating the effectiveness of the present RNAi strategy. This example highlights the capabilities of RNAi strategies as new therapeutic antidepressant class overcoming the two main limitations of current pharmacological treatments, that is, limited efficacy and slowness of action.

Given the growing implication of RNAi pathway in the pathophysiology and treatment of psychiatric disorders and the role played by 5-HT in these disorders, the relationships between RNAi and serotonergic neurotransmission are of interest. Indeed, various 5-HT receptors play a critical role in major depressive disorder and antidepressant effects (see Part I). In particular, 5-HT_{1A} receptors (both pre- and postsynaptic) play a major role in determining antidepressant responses. Hence, individuals with high 5-HT_{1A} autoreceptor expression, -including those with a functional polymorphism in the promoter region of Htr1a gene-, are more vulnerable to depression and suicide and respond poorly to current antidepressant treatments (Stockmeier et al., 1998). In addition, presynaptic 5-HT_{1A} autoreceptors play a detrimental role in the action of SSRI/SNRI since these drugs indirectly activate them, reducing serotonergic activity and serotonin release in forebrain (see Part I). Given the molecular identity of pre- and postsynaptic 5-HT_{1A} receptors, selective antagonists cannot discriminate between them and, unlike pindolol (Artigas et al., 2001), do not enhance nor accelerate the antidepressant effects of SSRI (Scorza et al., 2012).

The inability of pharmacological tools to selectively reduce presynaptic 5-HT_{1A} function (without altering that of postsynaptic 5-HT_{1A} receptors) led us to use siRNA tools. Like with SERT knockdown induced by conjugated siRNA, we developed a conjugated siRNA molecule in which the siRNA directed against the 5-HT_{1A} receptor mRNA was covalently bound to the SSRI sertraline, under the working hypothesis stated above. The conjugated siRNA was delivered to the mouse using intracerebroventricular or intranasal routes and produced a selective reduction of the expression of 5-HT_{1A} autoreceptors in the dorsal raphe, without affecting postsynaptic 5-HT_{1A} receptor expression, and evoking a marked antidepressant-like effect in the forced swim and tail suspension tests as well as increased 5-HT neurotransmission (Bortolozzi et al., 2012). Interestingly, the same effects, with a similar effect size, were also produced by the local (intra-raphé) administration of non-conjugated siRNA molecules directed against the 5-HT_{1A} receptor (Ferrés-Coy et al., 2013b). Overall, these observations suggest that the selective reduction of 5-HT_{1A} receptor expression may be novel antidepressant target by itself, without the need to block SERT. In resting -physiological- conditions, excitatory and inhibitory inputs are in equilibrium (including self-inhibitory mechanisms resulting from 5-HT_{1A} autoreceptor activation). This results in a slow and regular discharge of serotonergic neurons that determines 5-HT release in forebrain. In conditions of high excitatory inputs - such as during stress-, serotonergic neurons increase their local release in the raphe nuclei. The excess 5-HT activates 5-HT_{1A} autoreceptors, thus compensating the effect of excitatory inputs on neuronal activity and keeping the discharge rate low and regular. In a situation of reduced 5-HT_{1A}

autoreceptor response –such as after siRNA treatments- the above negative feedback mechanism is less effective, which allows serotonergic neurons to increase their discharge in front of excitatory inputs and to release more 5-HT in cortico-limbic areas involved in stress response. In support of this view, only siRNA-treated mice had an increased 5-HT release in mPFC, associated with a reduction of immobility during the tail-suspension test (Ferrés-Coy et al., 2013b)

More recently, the two pore domain potassium channel TASK3 has been identified as a new target in depression, and its antagonists might become effective antidepressants (Gotter et al., 2011). Indeed, the permanent deletion of TASK3 in mice markedly reduced REM sleep and evoked antidepressant-like effects in various behavioral tests (Gotter et al., 2011). Using siRNA strategies, we validated the role of TASK3 channel expressed in 5-HT and NA neurons of mice as a new target in antidepressant drug development (Ferrés-Coy et al., personal communication). Selective silencing of TASK3 channels, mainly in the serotonin neurons, improved 5-HT neurotransmission in the PFC possibly due to an increase of membrane resting potential as previously observed after TREK-1 gene deletion, another member of the two pore domain potassium channel family (Heurteaux et al., 2006). Therefore, the TASK3 knockdown mice evoked antidepressant-like effects in the tail suspension and novelty suppressed feeding tests as well as they showed increased BDNF mRNA levels and number of DCX-positive neurons –marked of neurogenesis- in the hippocampus (Ferrés-Coy et al., personal communication). Overall, these results support the validity of TASK3 channel expressed by 5-HT and NA neurons as a new target for the antidepressant drug development.

2.3 siRNAs and non-monoaminergic targets involved in MDD

Given the potential use of siRNAs to investigate the role of key genes involved in the pathophysiology and treatment of neuropsychiatric disorders, several studies have focused on RNAi-based regulation of different pathways not directly related to monoaminergic signaling. Below, we summarize some of these targets which include neuropeptides, metabotropic glutamate receptors and trophic factors among others, some of which lack appropriate pharmacology.

The dynorphins are endogenous opioid peptides that arise from the precursor protein prodynorphin, hypothesized to be involved in the regulation of negative mood states and the neuroplasticity associated with addiction (Carlezon et al., 2009). Using viral-mediated RNAi knockdown of prodynorphin in the rat nucleus accumbens, it was possible to reduce the depressive-

like behavior in the forced swim test, although the same treatment did not affect anxiety-like behavior in the elevated plus maze. The results suggested that dynorphin actions in a restricted area of the nucleus accumbens contributes to depressive-like behavior and may be involved in neuroadaptations associated to cocaine addiction (Cohen et al., 2014).

Two non-pharmacological interventions for depression, electroconvulsive therapy (ECT) and deep brain stimulation (DBS), are associated with an increased release of adenosine and stimulation of purinergic A1 receptors (A1R) (Bekar et al., 2008; Sadek et al., 2011). Moreover, the upregulation of A1R led to resilience in various depressive-like tests. Conversely, A1R knockout mice displayed an increased depressive-like behavior and were resistant to the antidepressant effects of sleep deprivation (SD). In addition, some antidepressant treatments increased homer1a expression in the mouse mPFC. Specific siRNA knockdown of homer1a in mPFC enhanced depressive-like behavior and prevented the antidepressant effects of A1R upregulation, SD, imipramine, and ketamine treatments. In contrast, viral overexpression of homer1a in the mPFC had antidepressant effects. Thus, the increased expression of homer1a has been suggested to be a final common pathway mediating the antidepressant effects of various antidepressant treatments (Serchov et al., 2015).

Another target of interest in cognition and emotional states is the metabotropic glutamate receptor 7 (mGluR7). This glutamate receptor is also the most widely distributed presynaptic mGluRs present in a broad range of synapses. A growing body of evidence suggests that mGluR7 is a key player in shaping synaptic responses at glutamatergic synapses as well as being a key regulator of inhibitory GABAergic transmission. Thus, the development of selective pharmacological and genetic tools has allowed for the unravelling of mGluR7 receptor function in a large variety of physiological and behavioural processes. Knockout mice and siRNA knockdown has pointed to a role of mGluR7 in anxiety, extinction of fear and aversion learning, spatial memory and hormonal response to stress (Fendt et al., 2008; O'Connor et al., 2010).

Likewise, the cholinergic signaling in the hippocampus regulates social stress resilience and anxiety- and depression-like behavior (Mineur et al., 2013). Using virally-delivered shRNAs targeting acetylcholinesterase (AChE) into the hippocampus, it was shown that the decreased AChE activity increased anxiety- and depression-like behaviors and these effects were reversed by systemic administration of SSRI fluoxetine. Overall, the abnormalities in the cholinergic system may be critical for the etiology of mood disorders and could represent an endophenotype of depression.

Hippocampal BDNF and neurogenesis play important roles in the pathophysiology and treatment of MDD (Santarelli et al., 2003). Postmortem analyses have reported decreased hippocampal BDNF levels in suicide victims and depressed individuals (Dwivedi et al., 2003). In addition, animal studies suggest a direct relationship between reduced hippocampal neurogenesis and depressive-like behavior. Nevertheless, there is no direct evidence that the actual reduction of the BDNF protein in specific brain sites can induce depressive-like behaviors or affect neurogenesis *in vivo*. Using BDNF knockdown by RNAi and lentiviral vectors injected into specific sub-regions of the hippocampus, Taliaz et al., (2010) showed that a reduction in BDNF expression in the dentate gyrus, but not the CA3 subfield, decreased neurogenesis and elicited depressive-like behaviors. Moreover, specific BDNF knockdown in the ventral subiculum induced anhedonia in rats. These findings provide substantial support for the neurotrophic hypothesis of depression and specify anatomical and neurochemical targets for potential antidepressant interventions.

Finally, other promising targets for the development of new pharmacotherapies for MDD include phosphodiesterase 4 (PDE4) (Wang et al., 2013) and calcineurin (Mineur et al., 2014). Hence, microinfusions of lentiviral vectors containing shRNA targeting long-form PDE4Ds into rat PFC evoked similar antidepressant-like and cognition-enhancing effects of the prototypical PDE4 inhibitor, rolipram. In the second study, data indicated that the local downregulation of calcineurin (calcium-dependent phosphatase) levels in the amygdala of mice using adeno-associated viral-delivered shRNAs targeting calcineurin increases measures of anxiety-like behavior and to some extent depression-like behaviors. Taken together, these findings may facilitate the development of new approaches for the treatment of MDD and related disorders using RNAi-induced gene silencing.

3. Concluding remarks

No previous treatment before ketamine had achieved such remarkable antidepressant actions after a single administration. These observations have raised an enormous interest in the neuropsychopharmacology community at preclinical and clinical settings in order to identify new targets in antidepressant drug development, in order to overcome the limitations of monoamine-based drugs, outlined above. Indeed, the clinical effects of ketamine challenge the suitability of pharmacological targets and mechanisms on which current antidepressant treatments are based, and indicate that antidepressant drugs more rapid and efficacious than monoamine reuptake blockers can be developed in the future.

On the other hand the use of RNAi strategies offers a series of advantages, such as the ability to target any protein-encoding mRNA with short RNAs –e.g., miRNAs or siRNAs–, avoiding unspecific drug actions. This strategy may be particularly useful to modulate the expression of proteins lacking appropriate pharmacological agents. However, the application of these strategies is severely compromised by two main limitations, such as the delivery of short RNAs to the brain and the selective targeting of the neuronal populations of interest. Our approach to deliver SSRI-conjugated siRNAs to 5-HT neurons through the intranasal route has proven successful in order to solve these two main problems, eliciting rapid and robust antidepressant-like effects in rodents, and showing its potential translational value.

Funding and Disclosure

Support from the following grants is acknowledged: SAF2015-68346-P, SAF2016-75797-R and Retos-Colaboración Subprograma RTC-2015-3309-1, MINECO co-financed by European Regional Development Fund - ERDF), UE (Ministry of Economy and Competitiveness and European Regional Development Fund), PI12/00156, PI16/00287 and PI13/01390 (Instituto de Salud Carlos III, co-financed by ERDF), and Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red de Salud Mental, CIBERSAM. Support from the Generalitat de Catalunya (2014 SGR798) is also acknowledged. The sponsors had no further role in the design of the review, in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. All authors revised critically the manuscript and they gave final approval. We thank María Jaramillo for secretarial assistance.

F.A. has received consulting honoraria from Lundbeck and he and P.C. are PI and Co-PI respectively of a grant from Lundbeck. He is also member of the scientific advisory board of Neurolix. F.A. and A.B. are co-inventors of a patent on conjugated RNAi molecules.

References

- Aan het Rot, M., Collins, K.A., Murrough, J.W., Perez, A.M., Reich, D.L., Charney, D.S., Mathew, S.J., 2010. Safety and efficacy of repeated dose intravenous ketamine for treatment resistant depression. *Biol. Psychiatry* 67, 139-145.
- Abdallah, C.G., Adams, T.G., Kelmendi, B., Esterlis, I., Sanacora, G., Krystal, J.H., 2016. Ketamine's mechanism of action: a path to rapid-acting antidepressants. *Depress Anxiety* 33, 689-97.
- Adams, B.W., Moghaddam, B., 2001. Effect of clozapine, haloperidol, or M100907 on phencyclidine-activated glutamate efflux in the prefrontal cortex. *Biol. Psychiatry* 50, 750-757.
- Akinfiresoye, L., Tizabi, Y., 2013. Antidepressant effects of AMPA and ketamine combination: role of hippocampal BDNF, synapsin, and mTOR. *Psychopharmacology*, 230, 291-298.
- Alural, B., Genc, S., Haggarty, S.J., 2016. Diagnostic and therapeutic potential of microRNAs in neuropsychiatric disorders: Past, present, and future. *Prog. Neuropsychopharmacol. Biol. Psychiatry*. 2016 Apr 9. pii: S0278-5846(16)30037-9. doi: 10.1016/j.pnpbp.2016.03.010. (Epub ahead of print).
- Amargós-Bosch, M., López-Gil, X., Artigas, F., Adell, A., 2006. Clozapine and olanzapine, but not haloperidol, suppress serotonin efflux in the medial prefrontal cortex elicited by phencyclidine and ketamine. *Int. J. Neuropsychopharmacol.* 9, 565-573.
- Artigas, F., Celada, P., Laruelle, M., Adell, A., 2001. How does pindolol improve antidepressant action? *Trends Pharmacol Sci.* 22, 224-228.
- Autry, A.E., Adachi, M., Nosyreva, E., Na, E.S., Los, M.F., Cheng, P.F., Kavalali, E.T., Monteggia, L.M., 2011. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* 475, 91-95.
- Bai, M., Zhu, X., Zhang, Y., Zhang, S., Zhang, L., Xue, L., Yi, J., Yao, S., Zhang, X., 2012. Abnormal hippocampal BDNF and miR-16 expression is associated with depression-like behaviors induced by stress during early life. *PLoS One* 7(10):e46921.
- Baudry, A., Mouillet-Richard, S., Schneider, B., Launay, J.M., Kellermann, O., 2010. miR-16 targets the serotonin transporter: a new facet for adaptive responses to antidepressants. *Science* 329, 1537-41.
- Becker, R., Braun, U., Schwarz, A.J., Gass, N., Schweiger, J.I., Weber-Fahr, W., Schenker, E., Spedding, M., von Hohenberg, C.C., Risterucci, C., Zang, Z., Grimm, O., Tost, H., Sartorius, A., Meyer-Lindenberg, A. 2016 Species-conserved reconfigurations of brain network topology induced by ketamine. *Transl Psychiatry*. e786.
- Behlke, M.A., 2006. Progress towards in vivo use of siRNAs. *Mol Ther.* 13, 644-670.

- Behlke, M.A., 2008. Chemical modification of siRNAs for in vivo use. *Oligonucleotides* 18, 305-319.
- Bekar, L., Libionka, W., Tian, G.F., Xu, Q., Torres, A., Wang, X., Lovatt, D., Williams, E., Takano, T., Schnermann, J., Bakos, R., Nedergaard, M., 2008. Adenosine is crucial for deep brain stimulation-mediated attenuation of tremor. *Nat. Med.* 14, 75-80.
- Berman, R.M., Cappiello, A., Anand, A., Oren, D.A., Heninger, G.R., Charney, D.S., Krystal, J.H., 2000. Antidepressant effects of ketamine in depressed patients. *Biol. Psychiatry* 47, 351-354.
- Bernstein, H-G., Meyer-Lotz, G., Dobrowolny, H., Bannier, J., Steiner, J., Walter, M., Bogerts, B., 2015. Reduced density of glutamine synthetase immunoreactive astrocytes in different cortical areas in major depression but not in bipolar I disorder. *Front. Cell. Neurosci.* 9, 273.
- Bjorkholm, C., Monteggia, L.M., 2016. BDNF-a key transducer of antidepressant effects. *Neuropharmacology* 102, 72-79.
- Bortolozzi, A., Castañé, A., Semakova, J., Santana, N., Alvarado, G., Cortés, R., Ferrés-Coy, A., Fernández, G., Carmona, M.C., Toth, M., Perales, J.C., Montefeltro, A., Artigas, F., 2012. Selective siRNA-mediated suppression of 5-HT_{1A} autoreceptors evokes strong anti-depressant-like effects. *Mol. Psychiatry* 17, 612-623.
- Bortolozzi, A., Celada, P., Artigas, F., 2014. Novel therapeutic strategies in major depression: focus on RNAi and ketamine. *Curr. Pharm. Des.* 20: 3848-3860
- Carlezon, W.A. Jr, Béguin, C., Knoll, A.T., Cohen, B.M., 2009. Kappa-opioid ligands in the study and treatment of mood disorders. *Pharmacol. Ther.* 123, 334-343.
- Chalk, A.M., Wahlestedt, C., Sonnhhammer, E.L., 2004. Improved and automated prediction of effective siRNA. *Biochem. Biophys. Res. Commun.* 319, 264-274.
- Cheng, L.C., Pastrana, E., Tavazoie, M., Doetsch, F., 2009. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat. Neurosci.* 12, 399-408.
- Choi, M., Lee, S.H., Wang, S.E., Ko, S.Y., Song, M., Choi, J.S., Kim, Y.S., Duman, R.S., Son, H., 2015. Ketamine produces antidepressant-like effects through phosphorylation-dependent nuclear export of histone deacetylase 5 (HDAC5) in rats. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15755-15760.
- Choi, P.S., Zakhary, L., Choi, W.Y., Caron, S., Alvarez-Saavedra, E., Miska, E.A., McManus, M., Harfe, B., Giraldez, A.J., Horvitz, H.R., Schier, A.F., Dulac, C., 2008. Members of the miRNA-200 family regulate olfactory neurogenesis. *Neuron* 57, 41-55.
- Chowdhury, G.M., Behar, K.L., Cho, W., Thomas, M.A., Rothman, D.L., Sanacora, G., 2012. 1H-[13C]-nuclear magnetic resonance spectroscopy measures of ketamine's effect on amino acid neurotransmitter metabolism. *Biol. Psychiatry* 71, 1022-1025.

- Cohen, A., Whitfield, T.W., Kreifeldt, M., Koebel, P., Kieffer, B.L., Contet, C., George, O., Koob, G.F., 2014. Virus-mediated shRNA knockdown of prodynorphin in the rat nucleus accumbens attenuates depression-like behavior and cocaine locomotor sensitization. *PLoS One* 9, e97216.
- Covington, H.E., Lobo, M.K., Maze, I., Vialou, V., Hyman, J.M., Zaman, S., LaPlant, Q., Mouzon, E., Ghose, S., Tamminga, C.A., Neve, R.L., Deisseroth, K., Nestler, E.J., 2010. Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. *J. Neurosci.* 30, 16082-16090.
- Dawson, N., Morris, B.J., Pratt, J.A., 2013. Subanaesthetic Ketamine Treatment Alters Prefrontal Cortex Connectivity with thalamus and Ascending Subcortical Systems. *Schizophrenia Bull.* 39, 366-377.
- Doench, J.G., Petersen, C.P., Sharp, P.A., 2003. siRNAs can function as miRNAs. *Genes Dev.* 17, 438-442.
- Dougherty, D.D., Weiss, A.P., Cosgrove, R., Alpert, N.M., Cassem, E.H., Nierenberg, A.A., Price, B.H., Mayberg, H.S., Fischman, A.J., Rauch, S.L. 2003. Cerebral metabolic correlates as potential predictors of response to cingulotomy for major depression. *J. Neurosurg.* 99, 1010-1017.
- Drevets, W.C., Price, J.L., Simpson, J.R., Todd, R.D., Reich, T., Vannier, M., Raichle, M.E., 1997. Subgenual prefrontal abnormalities in mood disorders. *Nature* 386, 824-827.
- Duman, R.S., Aghajanian, G.K., Sanacora, G., Krystal, J.H., 2016. Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants. *Nat. Med.* 22, 238-249.
- Duncan, G.E., Moy, S.S., Knapp, D.J., Mueller, R.A., Breese, G.R., 1998. Metabolic mapping of the rat brain after subanesthetic doses of ketamine: potential relevance to schizophrenia. *Brain Res.* 787, 181-190.
- Dwivedi, Y., Rizavi, H.S., Conley, R.R., Roberts, R.C., Tamminga, C.A., Pandey, G.N., 2003. Altered gene expression of brain-derived neurotrophic factor receptor tyrosine kinase B in postmortem brain of suicide subjects. *Arch. Gen. Psychiatry* 60, 804-815.
- Dwyer, J.M., Lepack, A.E., Duman, R.S. 2012 mTOR activation is required for the antidepressant effects of mGluR2/3 blockade. *Int J Neuropsychopharmacology* 15, 429-434.
- Fabre, V., Boutrel, B., Hanoun, N., Lanfumey, L., Fattaccini, C.M., Demeneix, B., Adrien, J., Hamon, M., Martres, M.P., 2000. Homeostatic regulation of serotonergic function by the serotonin transporter as revealed by nonviral gene transfer. *J. Neurosci.* 20, 5065-5075.
- Fendt, M., Schmid, S., Thakker, D.R., Jacobson, L.H., Yamamoto, R., Mitsukawa, K., Maier, R., Natt, F., Hüsken, D., Kelly, P.H., McAllister, K.H., Hoyer, D., van der Putten, H., Cryan, J.F., Flor, P.J., 2008. mGluR7 facilitates extinction of aversive memories and controls amygdala plasticity. *Mol. Psychiatry* 13, 970-979.

Ferrés-Coy, A., Pilar-Cuellar, F., Vidal, R., Paz, V., Masana, M., Cortés, R., Carmona, M.C., Campa, L., Pazos, A., Montefeltro, A., Valdizán, E.M., Artigas, F., Bortolozzi, A., 2013a. RNAi-mediated serotonin transporter suppression rapidly increases serotonergic neurotransmission and hippocampal neurogenesis. *Transl. Psychiatry* 3, e211.

Ferrés-Coy, A., Santana, N., Castane, A., Cortés, R., Carmona, M.C., Toth, M., Montefeltro, A., Artigas, F., Bortolozzi, A., 2013b. Acute 5-HT(1)A autoreceptor knockdown increases antidepressant responses and serotonin release in stressful conditions. *Psychopharmacology (Berl)* 225, 61-74.

Ferrés-Coy, A., Galofré, M., Pilar-Cuellar, F., Vidal, R., Paz, V., Ruiz-Bronchal, E., Campa, L., Pazos, Á., Caso, J.R., Leza, J.C., Alvarado, G., Montefeltro, A., Valdizán, E.M., Artigas, F., Bortolozzi, A., 2016. Therapeutic antidepressant potential of a conjugated siRNA silencing the serotonin transporter after intranasal administration. *Mol. Psychiatry* 21, 328-338.

Ferguson, J.M., Shingleton, R.N., 2007. An open-label, flexible-dose study of memantine in major depressive disorder. *Clin. Neuropharmacol.* 30, 36-144.

Fuchikami, M., Thomas, A., Liu, R., Wohleb, E.S., Land, B.B., DiLeone, R.J., Aghajanian, G.K., Duman, R.S., 2015. Optogenetic stimulation of infralimbic PFC reproduces ketamine's rapid and sustained antidepressant actions. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8106-8111.

Fukumoto, K., Iijima, M., Chaki, S., 2016. The Antidepressant Effects of an mGlu2/3 Receptor Antagonist and Ketamine Require AMPA Receptor Stimulation in the mPFC and Subsequent Activation of the 5-HT Neurons in the DRN. *Neuropsychopharmacology* 41, 1046-1056.

Gaglione, M., Messere, A., 2010. Recent progress in chemically modified siRNAs. *Mini Rev. Med. Chem.* 10, 578-595.

Gasull-Camós, J., Tarrés-Gatius, M., Artigas, F., Castañé, A., 2017. Glial GLT-1 blockade in infralimbic cortex as a new strategy to evoke rapid antidepressant-like effects in rats. *Transl Psychiatry*. 7, (2):e1038.

Grimm, O., Gass, N., Weber-Fahr, W., Sartorius, A., Schenker E., Spedding M., Risterucci, C., Schweiger, J.I., Böhringer, A., Zang, Z., Tost, H., Schwarz, A.J., Meyer-Lindenberg, A., 2015. Acute ketamine challenge increases resting state prefrontal-hippocampal connectivity in both humans and rats. *Psychopharmacology (Berl)*. 232, 4231-4241.

Gotter, A.L., Santarelli, V.P., Doran, S.M., Tannenbaum, P.L., Kraus, R.L., Rosahl, T.W., Meziane, H., Montiel, M., Reiss, D.R., Wessner, K., McCampbell, A., Stevens, J., Brunner, J.I., Fox, S.V., Uebele, V.N., Bayliss, D.A., Winrow, C.J., Renger, J.J., 2011. TASK-3 as a potential antidepressant target. *Brain Res.* 1416:69-79.

Greicius, M.D., Flores, B.H., Menon, V., Glover, G.H., Solvason, H.B., Kenna, H., Reiss, A.L., Schatzberg, A.F., 2007. Resting-state functional connectivity in major depression: abnormally

increased contributions from subgenual cingulate cortex and thalamus. *Biol. Psychiatry*. 62, 429-437.

Hamani, C., Diwan, M., Macedo, C.E., Brandão, M.L., Shumake, J., Gonzalez-Lima, F., Raymond, R., Lozano, A.M., Fletcher, P.J., Nobrega, J.N., 2010. Antidepressant-like effects of medial prefrontal cortex deep brain stimulation in rats. *Biol. Psychiatry* 67, 117-124.

Hannon, G.J., 2002. RNA interference. *Nature* 418, 244-251.

Hanson, J.E., Weber, M., Meilandt, W.J., Wu, T., Luu, T., Deng, L., Shamloo, M., Sheng, M., Scarce-Levie, K., Zhou, Q., 2013. GluN2B antagonism affects interneurons and leads to immediate and persistent changes in synaptic plasticity, oscillations, and behavior. *Neuropsychopharmacology* 38, 1221-1233.

He, M., Liu, Y., Wang, X., Zhang, M.Q., Hannon, G.J., Huang, Z.J., 2012. Cell-type-based analysis of microRNA profiles in the mouse brain. *Neuron* 73, 35-48.

Heurteaux, C., Lucas, G., Guy, N., El Yacoubi, M., Thümmel, S., Peng, X.D., Noble, F., Blondeau, N., Widmann, C., Borsetto, M., Gobbi, G., Vaugeois, J.M., Debonnel, G., Lazdunski, M., 2006. Deletion of the background potassium channel TREK-1 results in a depression-resistant phenotype. *Nature Neurosci.* 9, 1134-1141.

Hogg, D.R., Harries, L.W., 2014. Human genetic variation and its effect on miRNA biogenesis, activity and function. *Biochem. Soc. Trans.* 42, 1184-1189.

Homayoun, H., Moghaddam, B., 2007 NMDA receptor hypofunction produces opposite effects on prefrontal cortex interneurons and pyramidal neurons. *J. Neurosci.* 27, 11496-11500.

Hoover, W.B., Vertes, R.P., 2007. Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Struct. Funct.* 212, 149-179.

Hornung, V., Guenther-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougères, A., Endres, S., Hartmann, G., 2005. Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* 11, 263-270.

Issler, O., Haramati, S., Paul, E.D., Maeno, H., Navon, I., Zwang, R., Gil, S., Mayberg, H.S., Dunlop, B.W., Menke, A., Awatramani, R., Binder, E.B., Deneris, E.S., Lowry, C.A., Chen, A., 2014. MicroRNA 135 is essential for chronic stress resiliency, antidepressant efficacy, and intact serotonergic activity. *Neuron* 83, 344-360.

Ji, G., Neugebauer, V., 2012. Modulation of medial prefrontal cortical activity using in vivo recordings and optogenetics. *Mol. Brain* 5, 36.

Jimenez-Sanchez, L., Campa, L., Auberson, Y.P., Adell, A., 2014. The Role of GluN2A and GluN2B Subunits on the effects of NMDA receptor antagonists in modeling schizophrenia and treating refractory depression . *Neuropsychopharmacology* 39, 2673-2680.

Jiménez-Sánchez, L., Castañé, A., Pérez-Caballero, L., Grifoll-Escoda, M., López-Gil, X., Campa, L., Galofré, M., Berrocoso, E., Adell, A., 2016. Activation of AMPA Receptors Mediates the Antidepressant Action of Deep Brain Stimulation of the Infralimbic Prefrontal Cortex. *Cereb. Cortex* 26, 2778-2789

Judge, A.D., Sood, V., Shaw, J.R., Fang, D., McClintock, K., MacLachlan, I., 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.* 23, 457-462.

Juliano, R., Alam, M.R., Dixit, V., Kang, H., 2008. Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. *Nucleic Acids Res.* 36, 4158-4171.

Kargieman, L., Santana, N., Mengod, G., Celada, P., Artigas, F., 2007. Antipsychotic drugs reverse the disruption in prefrontal cortex function produced by NMDA receptor blockade with phencyclidine. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14843-14848.

Kosik, K.S., 2006. The neuronal microRNA system. *Nat. Rev. Neurosci.* 7, 911-920.

Krol, J., Loedige, I., Filipowicz, W., 2010. The widespread regulation of microRNA biogenesis, function and decay. *Nat. Rev. Genet.* 11, 597-610.

Krystal, J.H., D'Souza, D.C., Mathalon, D., Perry, E., Belger, A., Hoffman, R., 2003. NMDA receptor antagonist effects, cortical glutamatergic function, and schizophrenia: toward a paradigm shift in medication development. *Psychopharmacology* 169, 215-233.

Larkin, G.L., Beautrais, A.L. 2011. A preliminary naturalistic study of low dose ketamine for depression and suicide ideation in the emergency department. *Int. J. Neuropsychopharmacol.* 14, 1127-1131.

Lau, C.G., Zukin, R.S., 2007. NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. *Nat. Rev. Neurosci.* 8, 413-426.

Launay, J.M., Mouillet-Richard, S., Baudry, A., Pietri, M., Kellermann, O., 2011. Raphe-mediated signals control the hippocampal response to SRI antidepressants via miR-16. *Transl Psychiatry.* 22;1:e56.

Lepack, A.E., Fuchikami, M., Dwyer, J.M., Banasr, M., Duman, R.S., ~~2015~~ 2014. BDNF release is required for the behavioral actions of ketamine. *Int. J. Neuropsychopharmacol.* 18(1). pii: pyu033. doi: 10.1093/ijnp/pyu033.

Li, C.T., Chen, M.H., Lin, W.C., Hong, C.J., Yang, B.H., Liu, R.S., Tu, P.C., Su, T.P., 2016. The effects of low-dose ketamine on the prefrontal cortex and amygdala in treatment-resistant depression: A randomized controlled study. *Hum. Brain Mapp.* 37, 1080-1090.

Li, N., Liu, R.J., Dwyer, J.M., Banasr, M., Lee, B., Son, H., Li, X.Y., Aghajanian, G., Duman, R.S., 2011. Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. *Biol. Psychiatry* 69, 754-761.

Li, N., Lee, B.Y., Liu, R.J., Banasr, M., Dwyer, J., Iwata, M., Li, X.Y., Aghajanian, G., Duman, R.S., 2010. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* 329, 959-964.

Li, M., Demenescu L.R., Colic, L., Metzger, C.D., Heinze, H-J., Steiner, J., Speck, O., Fejtova, A., Salvatore, G., Walter, M. 2017 Temporal Dynamics of Antidepressant Ketamine Effects on Glutamine Cycling Follow Regional Fingerprints of AMPA and NMDA Receptor Densities. *Neuropsychopharmacology* 42, 1201–1209.

Lin, S., Gregory, R.I., 2015. microRNA biogenesis pathways in cancer. *Nat. Rev. Cancer* 15, 321-333.

Liu, R.J., Fuchikami, M., Dwyer, J.M., Lepack, A.E., Duman, R.S., Aghajanian, G.K., 2013. GSK-3 inhibition potentiates the synaptogenic and antidepressant like effects of subthreshold doses of ketamine. *Neuropsychopharmacology* 38, 2268-2277.

Liu, R.J., Ota, K.T., Dutheil, S., Duman, R.S., Aghajanian, G.K., 2015. Ketamine strengthens CRF-activated amygdala inputs to basal dendrites in mPFC layer V pyramidal cells in the prelimbic but not infralimbic subregion, a key suppressor of stress responses. *Neuropsychopharmacology* 40, 2066-2075.

Lopez, J.P., Lim, R., Cruceanu, C., Crapper, L., Fasano, C., Labonte, B., Maussion, G., Yang, J.P., Yerko, V., Vigneault, E., El Mestikawy, S., Mechawar, N., Pavlidis, P., Turecki, G., 2014b. miR-1202 is a primate-specific and brain-enriched microRNA involved in major depression and antidepressant treatment. *Nat. Med.* 20, 764-768.

Makeyev, E.V., Zhang, J., Carrasco, M.A., Maniatis, T., 2007. The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol. Cell*, 27, 435-448.

Maltbie, E., Gopinath, K., Urushino, N., Kempf, D., Howell, L., 2016. Ketamine-induced brain activation in awake female nonhuman primates: a translational functional imaging model. *Psychopharmacology (Berl)*, 233, 961-972.

Maltbie, E.A., Kaundinya, G.S., Howell, L.L. Ketamine and pharmacological imaging: use of functional magnetic resonance imaging to evaluate mechanisms of action. 2017.Behav Pharmacol. 28, 610-622.

Mayberg, H.S., Brannan, S.K., Mahurin, R.K., Jerabek, P.A., Brickman, J.S., Tekell, J.L., Silva, A., McGinnis, S., Glass, T.G., Martin, C.C., Foxet P.T., 1997. Cingulate function in depression: a potential predictor of treatment response. Neuroreport 8, 1057-1061.

Mayberg, H.S., Lozano, A.M., Voon, V., McNeely, H.E., Seminowicz, D., Hamani, C., Schwalb, J. M., Kennedy, S.H., 2005. Deep brain stimulation for treatment-resistant depression. Neuron 45, 651-660.

Mayberg, H.S., Brannan, S.K., Tekell, J.L., Silva, J.A., Mahurin, R.K., McGinnis, S., Jerabek, P.A. 2000. Regional metabolic effects of fluoxetine in major depression: serial changes and relationship to clinical response. Biol. Psychiatry 48, 830-843.

Meister, G., Tuschl, T., 2004. Mechanisms of gene silencing by doublestranded RNA. Nature, 431, 343-349.

Mercer, T.R., Dinger, M.E., Mariani, J., Kosik, K.S., Mehler, M.F., Mattick, J.S., 2008. Noncoding RNAs in long-term memory formation. Neuroscientist. 14, 434-445.

Miller, O.H., Moran, J.T., Hall, B.J., 2016. Two cellular hypotheses explaining the initiation of ketamine's antidepressant actions: Direct inhibition and disinhibition. Neuropharmacology 100, 17-26.

Mineur, Y.S., Obayemi, A., Wigstrand, M.B., Fote, G.M., Calarco, C.A., Li, A.M., Picciotto, M.R., 2013. Cholinergic signaling in the hippocampus regulates social stress resilience and anxiety- and depression-like behavior. Proc. Natl. Acad. Sci. U.S.A. 110, 3573-3578.

Mineur, Y.S., Taylor, S.R., Picciotto, M.R., 2014. Calcineurin downregulation in the amygdala is sufficient to induce anxiety-like and depression-like behaviors in C57BL/6J male mice. Biol. Psychiatry 75, 991-998.

Mion, G., Villeveille, T., 2013. Ketamine pharmacology: an update (pharmacodynamics and molecular aspects, recent findings). CNS Neurosci. Ther. 19, 370-380.

Moaddel, R., Luckenbaugh, D.A., Xie, Y., Villaseñor, A., Brutsche, N.E., Machado-Vieira, R., Ramamoorthy, A., Lorenzo, M.P., Garcia, A., Bernier, M., Torjman, M.C., Barbas, C., Zarate, C.A. Jr, Wainer, I.W., 2015. D-serine plasma concentration is a potential biomarker of (R,S)-ketamine antidepressant response in subjects with treatment-resistant depression. Psychopharmacology (Berl). 232, 399-409.

Monteggia, L.M., Zarate, C. Jr., 2015. Antidepressant actions of ketamine: from molecular mechanisms to clinical practice. Curr. Opin. Neurobiol. 30:139-143.

- Murrough, J.W., Iosifescu, D.V., Chang, L.C., Al Jurdi, R.K., Green, C.E., Perez, A.M., Iqbal, S., Pillemer, S., Foulkes, A., Shah, A., Charney, D.S., Mathew, S.J., 2013. Antidepressant efficacy of ketamine in treatment-resistant major depression: a two-site randomized controlled trial. *Am. J. Psychiatry* 170, 1134-1142.
- Nestler, E.J., Peña, C.J., Kundakovic, M., Mitchell, A., Akbarian, S., 2015. Epigenetic Basis of Mental Illness. *Neuroscientist*. 2015 Oct 8. pii: 1073858415608147. (Epub ahead of print).
- Nosyreva, E., Szabla, K., Autry, A.E., Ryazanov, A.G., Monteggia, L.M., Kavalali, E.T., 2013. Acute suppression of spontaneous neurotransmission drives synaptic potentiation. *J. Neurosci.* 33, 6990-7002.
- O'Connor, R.M., Finger, B.C., Flor, P.J., Cryan, J.F., 2010. Metabotropic glutamate receptor 7: at the interface of cognition and emotion. *Eur. J. Pharmacol.* 639, 123-131.
- Ota, K.T., Duman, R.S., 2013. Environmental and pharmacological modulations of cellular plasticity: role in the pathophysiology and treatment of depression. *Neurobiol. Dis.* 57, 28-37.
- Ozcan, G., Ozpolat, B., Coleman, R.L., Sood, A.K., Lopez-Berestein, G., 2015. Preclinical and clinical development of siRNA-based therapeutics. *Adv. Drug Deliv. Rev.* 87, 108-119.
- Ozpolat, B., Sood, A.K., Lopez-Berestein, G., 2010. Nanomedicine based approaches for the delivery of siRNA in cancer. *J. Intern. Med.* 267, 44-53.
- Paddison, P.J., Caudy, A.A., Hannon, G.J., 2002. Stable suppression of gene expression by RNAi in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 99, 1443-1448.
- Paul, R., Schaaff, N., Padberg, F., Möller, H.J., Frodl, T., 2009. Comparison of racemic ketamine and S-ketamine in treatment-resistant major depression: report of two cases. *World J. Biol. Psychiatry* 10, 241-244.
- Peña, C.J., Bagot, R.C., Labonté, B., Nestler, E.J., 2014. Epigenetic signaling in psychiatric disorders. *J. Mol. Biol.* 426, 3389-3412.
- Pizzagalli, D.A., 2011. Frontocingulate dysfunction in depression: toward biomarkers of treatment response. *Neuropsychopharmacology* 36, 183-206.
- Pozzi, L., Pollak Dorocic, I., Wang, X., Carlén, M., Meletis, K., 2014. Mice lacking NMDA receptors in parvalbumin neurons display normal depression-related behavior and response to antidepressant action of NMDAR antagonists. *PLoS One* 9, e83879.
- Quirk, M.C., Sosulski, D.L., Feierstein, C.E., Uchida, N., Mainen, Z.F., 2009. A defined network of fast-spiking interneurons in orbitofrontal cortex: responses to behavioral contingencies and ketamine administration. *Front. Syst. Neurosci.* 3, 13.

Rettig, G.R., Behlke, M.A., 2012. Progress Toward In Vivo Use of siRNAs-II. *Molecular Therapy* 20, 483-512.

Réus, G.Z., Stringari, R.B., Kirsch, T.R., Fries, G.R., Kapczinski, F., Roesler, R., Quevedo, J., 2010. Neurochemical and behavioural effects of acute and chronic memantine administration in rats: further support for NMDA as a new pharmacological target for the treatment of depression? *Brain Res. Bull.* 81, 585–589.

Robbins, M., Judge, A., Ambegia, E., Choi, C., Yaworski, E., Palmer, L., McClintock, K., MacLachlan, I., 2008. Misinterpreting the therapeutic effects of small interfering RNA caused by immune stimulation. *Hum. Gene. Ther.* 19, 991-999.

Rowland, L.M., Bustillo, J.R., Mullins, P.G., Jung, R.E., Lenroot, R., Landgraf, E., Barrow, R., Yeo, R., Lauriello, J., Brooks, W.M., 2005. Effects of ketamine on anterior cingulate glutamate metabolism in healthy humans: A 4-T proton MRS study. *American J. Psychiatry* 162, 394-396.

Sadek, A.R., Knight, G.E., Burnstock, G., 2011. Electroconvulsive therapy: a novel hypothesis for the involvement of purinergic signalling. *Purinergic Signal.* 7, 447-452.

Salvadore, G., Cornwell, B.R., Colon-Rosario, V., Coppola, R., Grillon, C., Zarate, C.A. Jr, Manji, H.K., 2009. Increased anterior cingulate cortical activity in response to fearful faces: a neurophysiological biomarker that predicts rapid antidepressant response to ketamine. *Biol. Psychiatry* 65, 289-295.

Salvadore, G., Cornwell, B.R., Sambataro, F., Latov, D., Colon-Rosario, V., Carver, F., Holroyd, T., DiazGranados, N., Machado-Vieira, R., Grillon, C., Drevets, W.C., Zarate, C.A. Jr., 2010. Anterior cingulate desynchronization and functional connectivity with the amygdala during a working memory task predict rapid antidepressant response to ketamine. *Neuropsychopharmacology* 35, 1415-1422.

Santana, N., Troyano-Rodriguez, E., Mengod, G., Celada, P., Artigas, F., 2011. Activation of thalamocortical networks by the N-methyl-D-aspartate receptor antagonist phencyclidine: reversal by clozapine. *Biol. Psychiatry* 69, 918-927.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., Hen, R., 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301, 805-809.

Scheidegger, M., Walter, M., Lehmann, M., Metzger, C., Grimm, S., Boeker, H., Boesiger, P., Henning, A., Seifritz, E., 2012. Ketamine decreases resting state functional network connectivity in healthy subjects: implications for antidepressant drug action. *PLoS One* 7, e44799.

Schoevers, R.A., Chaves, T.V., Balukova, S.M., Rot, M.A., KorteKaas, R., 2016. Oral ketamine for the treatment of pain and treatment-resistant depression. *Br. J. Psychiatry* 208, 108-113.

Schratt, G.M., Tuebing, F., Nigh, E.A., Kane, C.G., Sabatini, M.E., Kiebler, M., Greenberg, M.E., 2006. A brain-specific microRNA regulates dendritic spine development. *Nature* 439, 283-289.

Scorza, M.C., Llado-Pelfort, L., Oller, S., Cortés, R., Puigdemont, D., Portella, M.J., Pérez-Egea, R., Alvarez, E., Celada, P., Pérez, V., Artigas, F., 2012. Preclinical and clinical characterization of the selective 5-HT(1A) receptor antagonist DU- 125530 for antidepressant treatment. *Br. J. Pharmacol.* 167, 1021-1034.

Serchov, T., Clement, H.W., Schwarz, M.K., Iasevoli, F., Tosh, D.K., Idzko, M., Jacobson, K.A., de Bartolomeis, A., Normann, C., Biber, K., van Calker, D., 2015. Increased Signaling via Adenosine A1 Receptors, Sleep Deprivation, Imipramine, and Ketamine Inhibit Depressive-like Behavior via Induction of Homer1a. *Neuron*. 87, 549-562.

Shishkina GT, Kalinina TS, Dygalo NN. Attenuation of alpha2Adrenergic receptor expression in neonatal rat brain by RNA interference or antisense oligonucleotide reduced anxiety in adulthood. *Neuroscience* 2004; 129: 521-8.

Skolnick, P., Popik, P., Trullas, R., 2009. Glutamate-based antidepressants: 20 years on. *Trends Pharmacol. Sci.* 30, 563-569.

Smalheiser, N.R., 2014. The RNA-centered view of the synapse: non-coding RNAs and synaptic plasticity. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369(1652).

Snow O, Jr., Hole, T., 2004. Many commonly used siRNAs risk off-target activity. *Biochem. Biophys. Res. Commun.* 319, 256-263.

Song, E., Zhu, P., Lee, S.K., Chowdhury, D., Kussman, S., Dykxhoorn, D.M., Feng, Y., Palliser, D., Weiner, D.B., Shankar, P., Marasco, W.A., Lieberman, J., 2005. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* 23, 709-717.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R.K., Racie, T., Rajeev, K.G., Rohl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M., Vornlocher, H.P., 2004. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173-178.

Stegmeier, F., Hu, G., Rickles, R.J., Hannon, G.J., Elledge, S.J., 2005. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13212-13217.

Stockmeier, C.A., Shapiro, L.A., Dilley, G.E., Kolli, T.N., Friedman, L., Rajkowska, G., 1998. Increase in serotonin- 1A autoreceptors in the midbrain of suicide victims with major depression-postmortem evidence for decreased serotonin activity. *J. Neurosci.* 18, 7394-7401.

Sun, H., Kennedy, P.J., Nestler, E.J., 2013. Epigenetics of the depressed brain: role of histone acetylation and methylation. *Neuropsychopharmacology*, 38, 124-137.

- Sun, E., Shi, Y., 2015. MicroRNAs: small molecules with big roles in neurodevelopment and diseases. *Exp. Neurol.* 268, 46-53.
- Sun, Y., Luo, Z.M., Guo, X.M., Su, D.F., Liu, X., 2015. An updated role of microRNA-124 in central nervous system disorders: a review. *Front. Cell Neurosci.* 9, 193.
- Taliaz, D., Stall, N., Dar, D.E., Zangen, A., 2010. Knockdown of brain-derived neurotrophic factor in specific brain sites precipitates behaviors associated with depression and reduces neurogenesis. *Mol. Psychiatry* 15, 80-92.
- Tang, G., 2005. siRNA and miRNA: an insight into RISCs. *Trends Biochem. Sci.* 30, 106-114.
- Thakker, D.R., Natt, F., Husken, D., Maier, R., Müller, M., van der Putten, H., Hoyer, D., Cryan, J.F., 2004. Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. *Proc. Natl. Acad. Sci. U.S.A.* 101, 17270-17275.
- Thakker, D.R., Natt, F., Husken, D., van der Putten, H., Maier, R., Hoyer, D., Cryan, J.F., 2005. siRNA-mediated knockdown of the serotonin transporter in the adult mouse brain. *Mol. Psychiatry* 10, 782-789.
- Trivedi, M.H., Fava, M., Wisniewski, S.R., Thase, M.E., Quitkin, F., Warden, D., Ritz, L., Nierenberg, A.A., Lebowitz, B.D., Biggs, M.M., Luther, J.F., Shores-Wilson, K., Rush, A.J., STAR*D Study Team, 2006. Medication augmentation after the failure of SSRIs for depression. *N. Engl. J. Med.* 354, 1243-1252.
- Troyano-Rodriguez, E., Lladó-Pelfort, L., Santana, N., Teruel-Martí, V., Celada, P., Artigas, F., 2014. Phencyclidine inhibits the activity of thalamic reticular gamma-aminobutyric acidergic neurons in rat brain. *Biol. Psychiatry* 76, 937-945.
- Tsankova, N., Berton O., Renthal, W., Kumar, A., Neve, R. L., Nestler, E.J., 2006. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* 9, 519–525.
- Vaisinen, J., Ihalainen, J., Tanila, H., Castren, E., 2004 Effects of NMDA-receptor antagonist treatment on c-fos expression in rat brain areas implicated in schizophrenia. *Cell. Mol. Neurobiol.* 24, 769-80.
- Villaseñor, A., Ramamoorthy, A., Silva dos Santos, M., Lorenzo, M.P., Laje, G., Zarate, C.A. Jr., Barbas, C., Wainer, I.W., 2014. A pilot study of plasma metabolomic patterns from patients treated with ketamine for bipolar depression: evidence for a response-related difference in mitochondrial networks. *Br. J. Pharmacol.* 171, 2230-2242.
- Vo, N., Klein, M.E., Varlamova, O., Keller, D.M., Yamamoto, T., Goodman, R.H., Impey, S., 2005. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 16426-16431.

- Vollenweider, F.X., Komater, M., 2010. The neurobiology of psychedelic drugs: implications for the treatment of mood disorders. *Nature Reviews Neuroscience* 11, 642-651.
- Vollenweider, F.X., Leenders, K.L., Oye, I., Hell, D., Angst, J., 1997. Differential psychopathology and patterns of cerebral glucose utilisation produced by (S)- and (R)-ketamine in healthy volunteers using positron emission tomography (PET). *Eur. Neuropsychopharmacol.* 7, 25-38.
- Vornlocher, H.P., 2006. Antibody-directed cell-type-specific delivery of siRNA. *Trends Mol. Med.* 12, 1-3.
- Wan, Y., Liu, Y., Wang, X., Wu, J., Liu, K., Zhou, J., Liu, L., Zhang, C., 2015. Identification of differential microRNAs in cerebrospinal fluid and serum of patients with major depressive disorder. *PLoS One* 10(3), e0121975.
- Wang, X., Sundquist, K., Hedelius, A., Palmér, K., Memon, A.A., Sundquist, J., 2015. Circulating microRNA-144-5p is associated with depressive disorders. *Clin. Epigenetics* 7, 69.
- Wang, Z.Z., Zhang, Y., Liu, Y.Q., Zhao, N., Zhang, Y.Z., Yuan, L., An, L., Li, J., Wang, X.Y., Qin, J.J., Wilson, S.P., O'Donnell, J.M., Zhang, H.T., Li, Y.F., 2013. RNA interference-mediated phosphodiesterase 4D splice variants knock-down in the prefrontal cortex produces antidepressant-like and cognition-enhancing effects. *Br. J. Pharmacol.* 168, 1001-1014.
- Warden, M.R., Selimbeyoglu, A., Mirzabekov, J.J., Lo, M., Thompson, K.R., Kim, S.Y., Adhikari, A., Tye, K.M., Frank, L.M., Deisseroth, K., 2012. A prefrontal cortex-brainstem neuronal projection that controls response to behavioural challenge. *Nature* 492, 428-432.
- Yang, X., Yang, Q., Wang, X., Luo, C., Wan, Y., Li, J., Liu, K., Zhou, M., Zhang, C., 2014. MicroRNA expression profile and functional analysis reveal that miR-206 is a critical novel gene for the expression of BDNF induced by ketamine. *Neuromolecular Med.* 16, 594-605.
- Zanos, P., Moaddel, R., Morris, P.J., Georgiou, P., Fischell, J., Elmer, G.I., Alkondon, M., Yuan, P., Pribut, H.J., Singh, N.S., Dossou, K.S., Fang, Y., Huang, X.P., Mayo, C.L., Wainer, I.W., Albuquerque, E.X., Thompson, S.M., Thomas, C.J., Zarate, C.A. Jr., Gould, T.D., 2016. NMDAR inhibition-independent antidepressant actions of ketamine metabolites. *Nature* 533, 481-486.
- Zarate, C.A. Jr., Singh, J.B., Carlson, P.J., Brutsche, N.E., Ameli, R., Luckenbaugh, D.A., Charney, D.S., Manji, H.K., 2006. A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Arch. Gen. Psychiatry* 63, 856-864.
- Zarate, C.A. Jr., Mathews, D., Ibrahim, L., Chaves, J.F., Marquardt, C., Ukoh, I., Jolkovsky, L., Brutsche, N.E., Smith, M.A., Luckenbaugh, D.A., 2013. A randomised trial of a low- trapping nonselective N-methyl-D-aspartate channel blocker in major depression. *Biol. Psychiatry* 74, 257-264.
- Zhang, J., Li, S., Hashimoto, K.R., 2014. R (-)-ketamine shows greater potency and longer lasting antidepressant effects than S (+)-ketamine. *Pharmacol. Biochem. Behav.* 116, 137-141.

Zhou, R., Yuan, P., Wang, Y., Hunsberger, J.G., Elkahloun, A., Wei, Y., Damschroder-Williams, P., Du, J., Chen, G., Manji, H.K., 2009. Evidence for selective microRNAs and their effectors as common long-term targets for the actions of mood stabilizers. *Neuropsychopharmacology* 34, 1395-1405.

Figures

Figure 1. . A. Number of documents retrieved from the *ISI Web of Science* with the word "ketamine" in the title since 2000. The graph in **B** shows the subgroup of documents relative to the antidepressant properties of ketamine since the first report on its antidepressant properties, in 2000 (Berman et al., 2000). Note the dramatic increase in publications during the last 5 6 yr.

Figure 2. Scheme showing the putative mechanism of the antidepressant action of the non-competitive NMDA receptor antagonist ketamine. Ketamine may increase excitatory neurotransmission in forebrain, and particularly in the prefrontal cortex (PFC) by blocking NMDA receptors in brain GABAergic elements, including fast spiking GABAergic interneurons and/or subcortical GABA elements, as observed for phencyclidine (Troyano-Rodríguez et al., 2014). In addition, the ketamine metabolite (2S,6S;2R,6R)-hydroxynorketamine acts a direct AMPA receptor agonist, thus contributing to the enhanced activation of AMPA receptors in PFC. This effect results in an increased mTOR signalling, increased synaptogenesis and an enhanced connectivity between the PFC and 5-HT neurons of the midbrain raphe nuclei which, in the end, enhances forebrain serotonergic neurotransmission (see text for a detailed explanation and references)-.

Figure 3. Schematic representation of a simplified model for the RNAi pathway. RNAs are transcribed in the nucleus in the form of a precursor (pri-miRNA), which is processed by Drosha into pre-miRNA. In this process, Drosha forms complexes with the DGCR8 protein. The pre-miRNA is exported out of the nucleus into the cytoplasm by Exportin-5 and cleaved by the enzyme Dicer to form the functional miRNA, which then combines with Argonaut (Ago) to form miRNA-ribonucleoprotein complex. The actual effector of the RNAi is the ribonucleoprotein complex RISC (RNA-induced silencing complex). The miRNA can either cause endonucleolytic cleavage of the target mRNA through Ago2 or blocks translational in the case of partial complementarity. In addition, after uptake of the exogenous synthesized siRNAs into the cells, they are loaded onto the RISC by RLC (RLC: RISC-loading complex), a process also involving the removal of the sense strand. The antisense strand guides the RISC to the complementary target mRNA, which is cleaved by the Ago2 protein. The inhibition of gene expression can be accomplished when a shRNA reaches the intracellular compartment after its extracellular application. Adapted from Bortolozzi et al., 2014.

Figure 4. New antidepressant strategy based on the silencing of SERT expression induced by a conjugated siRNA selectively targeting SERT in 5-HT neurons. The double-strand conjugated SERT siRNA contains a sertraline (SSRI) molecule covalently bound to the 5' end of the sense strand to confer cellular specificity. It was hypothesized that the presence of sertraline would allow the enrichment of the conjugated siRNA around the dense network of 5-HT axons. A polyethylene glycol was also bound to the 5' end of the antisense strand to protect the conjugated siRNA from RNases. Mice were treated with: phosphate-buffered saline (PBS) or sertraline-conjugated SERT-siRNA (Conj. SERT-siRNA) at 30 µg/day during 7 days treatment by intranasal route (i.n.). Other groups of mice were treated with saline or SSRI fluoxetine at 10 mg/kg/day, intraperitoneally (i.p.), during 7 or 28 days. Different variables predictive of clinical antidepressant action were assessed, as follows. **(A-B)** RNAi-induced SERT knockdown reduced 5-HT_{1A} autoreceptor function (A) and rapidly enhanced forebrain serotonin (5-HT) release (B). **(A)** Conj. SERT-siRNA (7 days) decreased 5-HT_{1A} receptor-mediated 8-OH-DPAT-stimulated [³⁵S]GTPγS binding in the dorsal raphe nucleus (DR), whereas fluoxetine (7 days) was without effect (n=3–4 mice/group; ***P<0.001 versus PBS-treated mice).

Fluoxetine reduced 5-HT_{1A}-autoreceptor function after 28 days treatment (n=3–4 mice/group; ***P<0.001 versus saline and fluoxetine 7days). **(B)** Intranasal conj. SERT-siRNA treatment increased extracellular 5-HT levels in caudate putamen (CPu) more rapidly than fluoxetine (n=4–10 mice/group; *P<0.05, **P<0.01, ***P<0.001 versus saline or PBS, respectively). **(C)** Conj. SERT-siRNA accelerates the proliferation of cellular precursors and dendrite complexity in the hippocampus. Representative images showing immature neurons identified with Neuro-D or doublecortin (DCX) markers. Short-term Conj. SERT-siRNA treatment (7 days) or chronic fluoxetine administration (28 days) increased the number of NeuroD-positive and DCX-positive neurons. At the left side, the images also show the traces from Sholl analysis of DCX-positive cells bearing a complex dendritic morphology in the dentate gyrus of mice in the different treatment groups. Scale bars: low = 100 μ m and high = 20 μ m. **(D-E)** Conj. SERT-siRNA efficiently attenuates the behavioral deficits in a stress-induced depression model. Grouped-housed male C57BL/6J mice were presented during 28 or 49 days with corticosterone in the presence or absence of an antidepressant treatment (Conj. SERT-siRNA 30 μ g/day, i.n. or fluoxetine 10 mg/kg/day, i.p.) during the last 7 or 28 days of the corticosterone regimen. **(D)** Short-term Conj. SERT-siRNA reversed the reduction of sucrose intake and preference in the corticosterone-induced anhedonia (n=10–16 mice/group; ***P<0.001 versus corticosterone stressed mice treated with PBS. Fluoxetine induced a similar recovery after 28 days, but not after 7 days, treatment (n=7–12 mice/group; ***P<0.001 versus corticosterone-stressed mice treated with saline). **(E)** Effect on novelty suppressed feeding test (NSFT). Seven-day Conj. SERT-siRNA, but not 7-day fluoxetine, reversed the increased latency to feed in corticosterone-treated mice (n=8–12 mice/group). Similar effects were elicited by 28-day fluoxetine administration (n=7–12 mice/group). Data are mean $\pm$ SEM. Adapted from Ferrés-Coy et al., 2016.

Figure 5. Selective siRNA-induced 5-HT_{1A} autoreceptor increase resilience to stressful conditions through an increase of the serotonergic tone. Mice were infused locally into dorsal raphe nucleus (DR) with: 1) artificial aCSF, 2) nonsense siRNA or 3) siRNA targets 5-HT_{1A} receptor (10 µg/day for 2 days). (A) Representative coronal brain sections showing the 5-HT_{1A}-siRNA-induced reduction of 5-HT_{1A} expression in DR at -4.36 mm anteroposterior coordinate taken from bregma. The boxes delimit areas of DR shown at higher magnification. Scale bars: low magnification = 2mm, high magnification = 500 µm. (B) Extracellular 5-HT levels were measured by in vivo microdialysis in medial prefrontal cortex (mPFC) of mice during acute stress induced by tail suspension test (TST). Note that 5-HT_{1A} autoreceptor knockdown mice showed a larger increase of 5-HT release in mPFC than control groups (n = 5–7 mice/group; **P<0.01 versus vehicle and nonsense-siRNA-treated mice). (C) In parallel, 5-HT_{1A}-siRNA- treated mice displayed a reduced immobility in the TST (n = 5–7 mice/group; *P<0.05 versus vehicle and nonsense-siRNA-treated mice). Values are mean ± SEM. Adapted from Ferrés-Coy et al., 2013b.