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Analysis of miRNome expression profile in hippocampus of rats treated with antidepressants

Doctorate thesis

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INTRODUCTION

1. Depression

According to the World Health Organization, major depression (MD) has become the second most prevalent cause of illness-induced disability worldwide and it will be the highest ranked cause of disease in the middle- and high-income countries by the year 2030 (Mathers and Loncar, 2006; Ferrari et al, 2010).

According to the Diagnostic and Statistical Manual of Mental disorders (DSM-V, 2013) the diagnosis of MD requires a distinct change of mood, characterized by sadness or irritability and accompanied by several psychophysiological changes, such as disturbance in sleep, appetite or sexual desire, anhedonia (loss of the ability to experience pleasure from natural rewards), diminished ability to think or concentrate, psychomotor agitation or retardation, and recurrent thoughts of death. These changes should last a minimum of 2 weeks and interfere considerably with work and family relations.

MD etiology is still unknown: it is a complex disorder characterized by the interaction between biological, genetic and environmental factors, and by a pathogenesis involving alterations in several biological systems. Currently, the symptoms of MD are hypothesized to result from a combination of inherent and environmental factors that destabilize the reciprocal interactions of multiple neural circuits. The prevalence is higher in woman (in the range of 1.5 to 2.5) and nearly 50% of the risk for depression is due to genetic factors (Fava and Kenneth, 2000). MD can be spontaneous, but often follows a traumatic emotional experience or can be a symptom of other diseases, most often neurological (i.e., stroke, multiple sclerosis, or Parkinson disease) or endocrine (Cushing's disease, hypothyroidism). MD can also be triggered or precipitated by pharmacological agents or drug abuse (Kenneson et al, 2013). The anatomical and

physiological basis of depression is still the subject of extensive investigation. Many brain region are involved in the pathology of MD: the limbic structures (in circuits involving the cingulate-hippocampus-mammillary bodies-anterior thalamus-cingulate), the reward circuits (Nucleus Accumbens, Amygdala, Ventral Tegmentum, Cingulate, Insula, Thalamus, Parahippocampal Gyrus and Prefrontal Cortex), hypothalamus and anterior temporal cortex. As a consequence, such diversity of underlying pathology combined with the complexity and plasticity of the central nervous system has confounded the development of effective pharmacological treatments for this disabling syndrome.

1.1 From monoaminergic hypothesis to neuroplasticity: the evolution of antidepressants

The most common pharmacological approach in the treatment of MD consists in the use of antidepressants (ADs).

The first hypothesis formulated on the pathogenesis of MD was the so-called "monoamine hypothesis of depression". This hypothesis speculates that depression is related to decreased levels of centrally available monoamines: noradrenaline, serotonin and dopamine. The hypothesis suggested a diminished availability of monoamines, and was based on the serendipitous discovery, in the late 1950s, that two structurally unrelated classes of compounds were effective in treating severe depression (Moncrieff, 2008). The first class of compounds was the irreversible Monoamine Oxidase Inhibitors (MAOI). MAOIs inhibit the enzyme MAO, involved in the catabolism

of biogenic amines (serotonin, noradrenaline and dopamine); moreover, they are also defined by their selectivity. Some of them selectively inhibit isoenzyme A (i.e. moclobemide), other selectively inhibit MAO-B (i.e. pargyline) and some are non-selective inhibitors of both A and B (i.e. phenelzine). MAOIs are associated with serious side effects (hypertensive crisis) and require dietary restriction; nowadays, these drugs should generally be reserved for patients who do not respond to other treatments. The second class of compounds was the tricyclic antidepressants (TCAs, including imipramine and desipramine) that inhibit serotonin and/or noradrenaline reuptake. In addition to their therapeutic effect via activity on monoaminergic systems, TCAs are antagonists at non-target receptors including histamine, muscarinic and α1-adrenergic receptors. The activity at these sites causes adverse effects including sedation, body weight gain, constipation, memory disorders and orthostatic hypotension.

The monoaminergic hypothesis in its original form is clearly inadequate: it does not provide a complete explanation for the actions of antidepressants, particularly their delayed onset of action. The hypothesis has evolved over the years to include, for example, adaptive changes in receptors to explain why there should be only a gradual clinical response to antidepressant treatment, when the increase in availability of monoamines is rapid. More recent years have seen the development of selective serotonin reuptake inhibitors (SSRIs, such as fluoxetine) and serotonin/noradrenaline reuptake inhibitors (SNRIs, such as venlafaxine). However, despite the improvement in side effects and drug interactions, these classes of drugs, similar to all ADs, take 2 to 6 weeks to become effective (Berton and Nestler, 2006). Additionally, approximately 30% to 45% of patients fail to attain an adequate response to their initial pharmacotherapy (Fava, 2000).

Another heterogeneous group of drugs is formed by the atypical ADs; these drugs also affect monoamine neurotransmitters, receptors, or transporters. This class includes drugs with different profiles, such as bupropion, whose mechanism of action remains elusive, as it is a weak dopamine and norepinephrine reuptake inhibitor, with a relatively favorable side effect profile (Holtzheimer and Nemeroff, 2006). The NASSA (Noradrenergic and Specific Serotonergic Antidepressants), such as mirtazapine and mianserin, that increase the release of noradrenaline and serotonin by blocking the ⍺-2 receptor, or reboxetine (a potent and selective inhibitor of the norepinephrine transporter, markedly inhibiting noradrenaline reuptake), are additional available therapeutic options (Schatzberg and Nemeroff, 2009).

Agomelatine is the latest antidepressant agent with a novel pharmacological profile due to its unique receptor binding; it is a potent selective agonist at melatonergic MT1/MT2 receptors and an antagonist at serotonergic 5-HT2C receptors. Its unique and novel pharmacological profile exerts antidepressant-like activity via regulation of circadian rhythms (Popoli, 2009).

Although in the last several years clinical and basic research studies have made significant progress towards deciphering the pathophysiological events in the brain involved in development, maintenance, and treatment of MD, currently available antidepressants, are effective in only a subset of MD patients (approximately one-third respond to the first medication prescribed, up to two-thirds with multiple drug prescriptions) and require long-term treatment (weeks to months) to achieve therapeutic response (Fava and Davidson, 1996; Little 2009).

The principal molecular and cellular events contributing to the etiology and treatment of MD are not well characterized, but recent developments in the field have addressed

different neural pathways involved in the etiology of depression. Evidence of depression-associated neuronal atrophy, cell loss, and reduced tissue volume occurring in human brains, combined with a robust decrease in neurotrophic factor levels in animal models of depression, has led to formation of the neurotrophic hypothesis of depression (Duman and Li, 2012), which identifies altered neurotrophic signaling as a key contributing factor, as well as neuronal plasticity promoted by antidepressant treatments. The present and updated version of the hypothesis, which is called the "hypothesis of neuroplasticity," integrates postreceptor intracellular signaling cascades with several other processes, including synaptic and neurotrophic mechanisms, neurogenesis and the mechanisms of gene expression, including epigenetic mechanisms (Racagni and Popoli 2008; Nestler 2014).

2. Epigenetics and microRNAs

Growing evidence supports the hypothesis that epigenetics is a key mechanism through which environmental exposures interact with an individual's genetic background to determine risk for depression throughout life (Vialou et al, 2013). The term 'epigenetics' refers to long-lasting changes in gene expression that cannot be accounted for by changes in DNA sequence and that can be influenced by the environment. Recent research has involved epigenetic mechanisms in memory formation and synaptic plasticity, adaptation/response to environmental stimuli (e.g., stress) and pathophysiology of psychiatric disorders, including MD (Mouillet-Richard et al 2012; Tardito et al, 2013). Three main epigenetic mechanisms widely studied at present are

DNA methylation, posttranslational modification of histone proteins and posttranscriptional regulation of gene expression, particularly microRNAs (miRNAs).

MiRNAs were first identified in classical genetic studies as regulators of developmental timing in Caenorabditis elegans (Lee et al, 1993). These short noncoding RNAs were then found in other organisms by virtue of striking sequence conservation across species. MiRNA genes are encoded within the genome, suggesting that their transcription might be tightly coordinated with the transcription of other genes, including the protein coding genes that serve either as a source of miRNAs or as their targets.

The localization of about 50% of mammalian miRNA-coding genes has been found to be in the intergenic space. Most of the intergenic miRNAs are autonomously expressed and possess their own enhancer and promoter elements (Corcoran et al, 2009; Lagos-Quintana et al 2001). Approximately 40% of miRNA genes are localized within gene introns (Rodriguez et al, 2004) and finally the last 10% of known miRNA genes are located within exons. Regardless of the genomic location, generation of mature miRNAs occurs in a highly conserved fashion that involves the processing of the primary miRNA transcript in the nucleus to the mature product in the cytosol (Figure 1). The majority of miRNAs are transcribed by RNA polymerase II to primary miRNA (pri-miRNA) transcripts, highly complex double-stranded stem loop structures of 100-1000 nucleotides in length. The pri-miRNA stem loop structures are recognized by the nuclear microprocessor complex that contains two core proteins, Drosha and DGCR8 (DiGeorge syndrome critical region 8). DGCR8 recognizes and binds to the stem region of the pri-miRNA hairpin followed by the recruitment of Drosha and ensuing cleavage of pri-miRNA and generation of the precursor-miRNA (pre-miRNA) (Denli et al, 2004; Gregory

et al, 2004). The nascent pre-miRNAs are exported to the cytoplasm by the karyopherin protein family member Exportin-5 in a GTP-dependent fashion (Bohnsack et al, 2004). Once in the cytoplasm, the pre-miRNAs are cleaved in a $\cong$ 20 bp miRNA/miRNA* duplex by the RNase-III type enzyme Dicer and its cofactor, the mammalian TAR RNA-binding protein (TRBP). In mammals, Dicer is supported by Argonaute 2 (Ago2), an RNaseH-like endonuclease that cleave the 3' arms of some pre-miRNAs. The 'guide' strand of the miRNA duplex is then loaded into the RNA-inducing silencing complex (RISC), whereas the other strand (miRNA*) is released and degraded, although in some cases both strands can associate with RISC to target distinct sets of mRNAs (Breving et al, 2010; Krol et al, 2010) (Figure 1). Within the RISC complex, composed of a set of well-known proteins and components whose functions have not been fully clarified, miRNAs interact with their targets through base pairing. The initial bases of miRNA sequences (bases 2-8), namely the seed sequences, are the only bases that generally pair with perfect complementarity to the target mRNA, mainly at the 3' untranslated region, and are responsible for definition of target specificity (Pasquinelli 2012). The RISC complex is involved in at least three different functions: inhibition of translation initiation and/or elongation, cotranslational protein degradation, premature termination of translation and mRNA deadenylation (resulting in mRNA degradation) (Krol et al, 2010; Huntzinger and Izaurralde 2011).
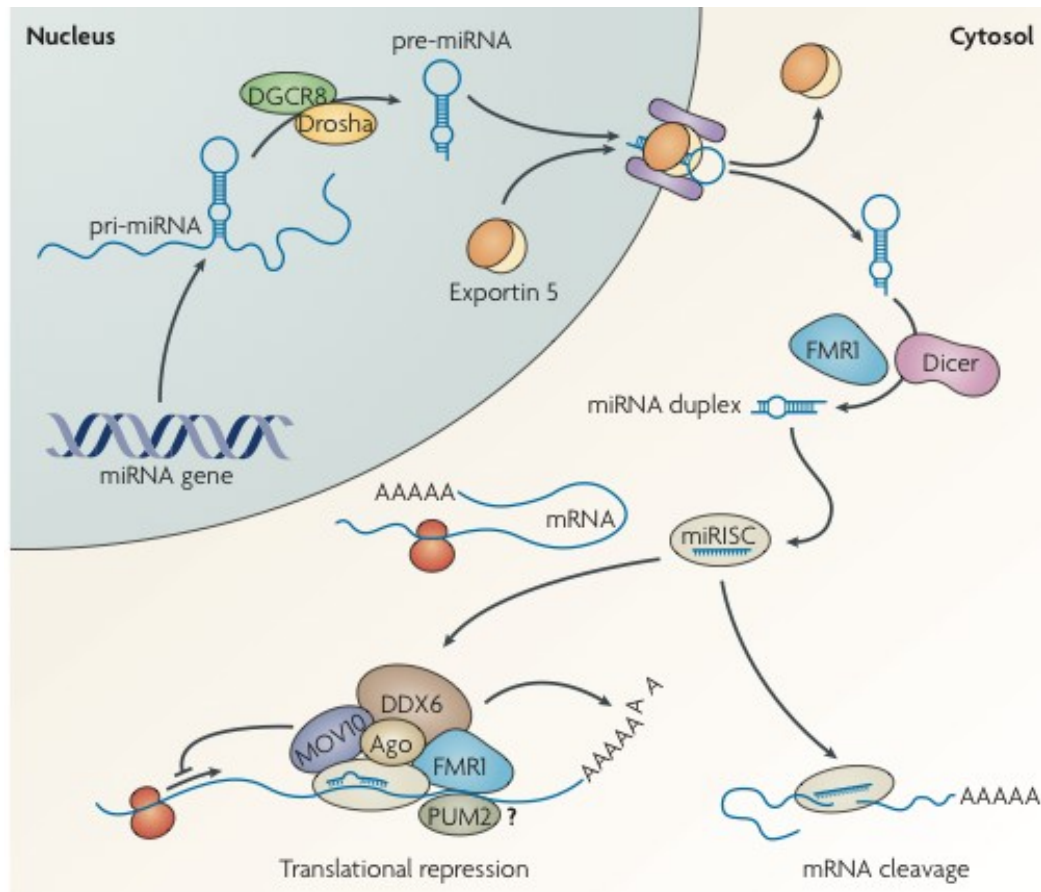
Figure 1: miRNA biogenesis pathway- adapted from Schratt et al, 2009

The processes described above are generally seen as the miRNA canonical aspects and their main mode of action. However, current literature has brought to light non-conventional miRNA features. For example, It is a general assumption that pre-miRNAs are transported by Exportin-5 in a one-way direction from nucleus to cytoplasm. However, many lines of evidence indicate that these molecules can also be guided back to the nucleus. CRM1 (Exportin-1), known to transport different classes of RNAs, enables the miRNA nuclear import (Castanotto et al., 2009). MiRNAs may enter the nucleus to undergo modifications, associate with nuclear proteins or with target transcripts, participate in chromatin remodeling, or regulate other non-coding RNAs. An example of

miRNAs directly regulating transcriptional silencing includes the knockdown of POLR3D mRNA expression due to increased levels of mature miR-320 (Kim et al, 2008).

Moreover, an additional mechanism of gene expression regulation through miRNA activity is emerging. MiRNAs have been recently proposed to recognize and guide transcription factors to their correct gene promoters (Korla et al, 2013), reinforcing the hypothesis that miRNAs may have an important role also in the nucleus. Positive regulation of gene expression is also an emerging feature of miRNAs at the posttranscriptional level. The first study to verify that miRNAs can act by up-regulating translation suggested a model by which human miR-369-3 directs, in a sequence-specific manner, the association of Ago and fragile X mental retardation-related protein 1 to the AU-rich element of the tumor necrosis factor-α (TNFα) mRNA under starvation conditions, leading to increased TNFα translation efficiency (Vasudevan et al, 2007). Similar results were found later and demonstrated that miR-10a targets the 5'UTR of ribosomal protein (RP) mRNAs, which results in enhanced translation of RP under aminoacid starvation. As a consequence, indirect global protein synthesis may also occurr through the aforementioned miRNA–mRNA interaction, since it leads to increased availability of the translational machinery (Ørometal??, 2008). Together, this data may suggest that positive or negative posttranscriptional regulation by miRNAs might take place depending on the physiological state of the cell or organism.

2.1 MiRNAs in the brain: role in pathophysiology and pharmacotherapy of MD

In the last few years, growing evidence has supported a key role for miRNAs in central nervous system (CNS) development and homeostasis. It has been reported that almost 50% of all identified miRNAs are expressed in mammalian brain, where they appear to be differentially distributed not only in specific areas but also within neurons and distinct neuronal compartments. This highly specific compartmentalization has been shown to be a key factor in the control of local protein expression, synapse development and function: in this regard, a role of miRNAs in the regulation of important aspects of synapse development and plasticity is indeed emerging. Even within a single neuron, complex functional architecture offers many compartments that could be regulated by different sets of miRNAs. It has been suggested that miRNAs might participate in the local and dynamic control of mRNA translation in dendrites; in particular, miR-184, is the prototypic dendritically localized miRNA that functions in dendritic protein synthesis in hippocampal neurons (Schratt et al 2006). Moreover, evidence has been recently provided that miRNAs might have a role in synapse maturation and growth. For example, in *D. melanogaster* let-7 family members are required for the maturation of the neuromuscular junction (Caygill and Johnston, 2008) and in mammals central neurons from Dicer-null mice show dramatic alterations in spine morphology, suggesting that miRNAs are crucial for the maturation of synaptic contacts (Davis et al, 2008). MiRNAs have also been implicated in the control of synaptic plasticity, learning and memory: in *D. melanogaster*, mutations in Dicer and Armitage, an helicase required for miRNA-mediated gene silencing, led to impaired memory formation during olfactory learning (Ashraf 2006). Intriguingly, the underlying defect probably involves aberrant synaptic

synthesis of crucial regulatory proteins, such as CAMKIIα, in these mutants. The storage of memories coincides with proteasome-mediated degradation of Armitage, suggesting that synaptic activation might overcome the block of synaptic protein synthesis imposed by miRNA-associated protein complexes.

Synapses are increasingly recognized as central structures in the etiology of a number of neurological and psychiatry disorders. The fine-tuning miRNAs expression profile, their unique mode of functioning (the ability of a single miRNA to target several different mRNAs often belonging to specific functional networks) and the knowledge of miRNA functions in all steps in synapse development has prompted research toward the study of potential involvement of miRNAs in the pathogenesis and pharmacotherapy of neuropsychiatric disorders, including MD. Numerous preclinical investigations showed that different stress paradigms might influence the expression of miRNAs and some of their targets in different brain areas, thus further supporting an involvement of miRNAs in MD. For example, miR-34c was found to be markedly upregulated after acute and chronic stress in mice and downregulated in Dicer ablated cells (Haramati et al 2011). An overall decrease in miRNA expression was observed in prefrontal cortex of depressed suicides, with significant modifications in 21 miRNAs. Some of the predicted targets for altered miRNAs were Vascular Endotelial Growth Factor receptor, Neuropilin-1, Growth-associated Protein 43, Synaptosomal associated protein 25, synaptojanin-1, synaptotagmin-1, Lim kinase-1, B-cell lymphoma 2 and DNA methyl-transferase 3b. In addition, other signaling proteins, ion channels, and ubiquitin ligases were identified. Several of these proteins have been previously linked to MD, thus suggesting a role for miRNAs in the pathogenesis of this disease (Smalheiser et al 2012). Further support for involvement of miRNAs in MD was given by studies investigating the presence of

genetic variations in miRNA genes or in miRNA processing genes in affected patients; for example, the ss178077483 polymorphism in the pre-miRNA-30e was positively associated with major depressive disorder risk (Xu et al 2010). Moreover, a recent study from Lopez et al (2014) has shown, by means of complementary studies using postmortem human brain samples, cellular assays and samples from clinical trials of patients with depression, a differential expression of miR-1202, a miRNA specific to primates and enriched in the human brain, in individuals with depression. Additionally, the authors also demonstrate that miR-1202 regulates expression of the gene encoding metabotropic glutamate receptor-4 and predicts antidepressant response at baseline thus suggesting that miR-1202 could be associated with the pathophysiology of depression.

MiRNAs have also been involved in the action of psychotropic drugs. The first evidence was provided by Zhou et al (2009), who showed that chronic treatment with the mood stabilizers lithium or valproate induced significant modifications in the expression levels of a number of miRNAs in rat hippocampus. Baudry et al (2010) were the first to demonstrate a possible role for miRNAs in the action of antidepressants. It was reported that miR-16, which among other targets also regulates the serotonin transporter (SERT), is expressed at higher levels in noradrenergic vs serotonergic cells; its reduction in noradrenergic neurons causes de novo SERT expression. In mice, prolonged exposure to fluoxetine, whose primary mechanism of action is related to the inhibition of SERT, increased miR-16 levels in serotonergic raphe nuclei, which in turn reduced SERT expression. This data suggest a role for miR-16 in the therapeutic action of SSRI antidepressants, creating new serotonin sources through the switch of noradrenergic neurons toward a serotonergic phenotype. In this context,

antidepressant effects on miRNAs expression in depressed patients was recently investigated in a clinical study that reported changes in blood miRNA expression induced by 12 weeks of treatment with the SSRI escitalopram in depressed patients. Out of 755 analyzed, 30 miRNAs were differentially expressed after treatment (28 miRNAs upregulated and 2 downregulated). Many of these miRNAs have a key role in neuroplasticity and stress response and some of them were previously associated with the pathogenesis of psychiatric disorders and the mechanism of action of psychotropic drugs (i.e., let-7d, let-7e, miR-26a, miR-26b, miR-34c-5p, miR-128, miR-132, miR-494 and miR-22*). MiRNA targets prediction, and functional annotation analysis showed a significant enrichment in several pathways associated with neuronal function (such as neuroactive ligand-receptor interaction, axon guidance, long-term potentiation and depression), supporting the hypothesis that these miRNAs may be involved in antidepressant action (Bocchio-Chiavetto et al, 2012) This hypothesis was suggested also by another study by O'Connor et al (2013), in which changes in rat hippocampal miRNA expression were found after treatment with acute ketamine (an NMDA receptor antagonist shown to induce a rapid and persistent antidepressant effect), electroconvulsive shock therapy, and fluoxetine.

To date, the actual experimental evidence for involvement of miRNAs in the pathophysiology and treatment of depression remains scarce. The involvement of miRNAs in the diverse pathways known to be associated with depression suggests that restoration of efficient neurogenesis, neurotransmission, structural and functional brain plasticity as well as circadian rhythms upon antidepressant treatment is likely to require a concerted miRNA response. The challenge now is to reach an integrated view of the network of miRNAs that are mobilized by antidepressants and the identification of

functionally relevant targets of these miRNAs because this should help to unravel the global molecular and cellular changes sustaining the therapeutic action of antidepressants. Deciphering the step-wise changes in the expression of appropriate miRNAs should eventually help identify the molecular events that trigger recovery from depression.

CHAPTER I

Early and time-dependent effects of antidepressants on hippocampal miRNome

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Abstract

MicroRNAs (miRNAs) have recently emerged as key regulators of complex temporal and spatial patterns of gene/protein expression changes in the brain, where they have a crucial role in the regulation of neurogenesis, neuronal differentiation and survival, as well as in neuroplasticity. Studies have begun to show that miRNAs could be involved in the pathophysiology of mood disorders and in the mechanism of action of some psychotropic drugs, but much remains to be uncovered. Here we investigated whether and how short-term treatments (for 3 and 7 days) with two different antidepressants, desipramine and fluoxetine, could affect rat hippocampal miRNome. We found that, although in a specific way, both antidepressants induced early and time-dependent modifications in miRNA expression. Indeed, desipramine increased the levels of eight miRNAs after three days of treatment and of thirteen additional miRNAs after 7 days. On the other hand, fluoxetine downregulated eight miRNAs after 3 days and changed the expression of thirty-five miRNAs (seven reduced and twenty-eight increased) after 7 days of treatment. Interestingly, after seven days of treatment, eight miRNAs were similarly modulated by both drugs, thus suggesting a common action on hippocampal miRNome. Bioinformatic analysis highlighted significant enrichment of miRNA targets in different pathways, many of which related to neuronal functions previously associated to both mood disorders pathophysiology and antidepressant action. Moreover, we validated changes in the expression of some putative target genes, such as Bmp7, Galr1, Kv4.2, Smad2 and Tgfbr1, that could be of interest in unraveling antidepressant mechanism of action.

Overall, our data support a role for miRNAs as early mediators of antidepressant effects and open new windows in the research for better therapies for mood disorders.

Introduction

Although antidepressant drugs are beneficial for many patients affected by mood and anxiety disorders, current pharmacological interventions still have many limitations, concerning mainly the slow onset of action and the lack of response in approximately 30-40% of subjects (1). One of the main reasons for that is that most available antidepressants are still historically based mainly on the assumption of underlying monoaminergic dysfunctions. Although much remains to be clarified, it is now understood that multiple complex alterations in structural and functional neuronal plasticity underlie the pathophysiology of these disorders (2-6). In this context, recent molecular, cellular, and behavioral findings have begun to reveal the importance of epigenetic mechanisms, dynamically regulating patterns of gene expression, that may play a critical role in the pathophysiology and pharmacotherapy of mood disorders (7-10). Epigenetics refers to the regulation of transcription and translation without alteration of the original DNA sequence and is controlled by DNA methylation, histone posttranslational modifications and non-coding RNAs, such as microRNAs (miRNAs). miRNAs are a large family of conserved small (20–22 nucleotides) non-coding RNAs, with a key role in the post-transcriptional regulation of gene expression. miRNAs, which are directly connected to the epigenetic machinery through a regulatory loop (11), can regulate entire genetic circuitries by modulating stability and/or translation of target mRNAs in a coordinated way through an interaction that generally takes place between the 5'-end of the miRNA and the 3'untranslated region (UTR) of the target mRNA (12, 13). Recent lines of evidence suggest that besides the canonical action of inhibiting protein expression, miRNAs can also lead to increased protein translation (9, 14, 15). In mammals, miRNAs, predicted to control the activity of ~50% of all the protein-coding genes, are involved in virtually all biological functions and in several human diseases (16, 17), and are particularly abundant in the brain where they participate to both

neuronal development and function (18-21). Indeed, it has been shown that miRNAs have a key role in the regulation of neurogenesis, neuronal differentiation and survival, as well as in neuronal and synaptic plasticity (20, 22-24), processes tightly associated with both the pathophysiology and pharmacotherapy of mood disorders. In this regard, altered profiles of miRNA expression have been reported in both post mortem brain and peripheral tissues of patients with mood disorders (for reviews see 9 and 25; 26-29). Moreover, a role for miRNAs in the pharmacotherapy of mood disorders has been suggested by both preclinical and clinical studies (9, 30). In particular, regarding antidepressants, changes in miRNA expression were reported in blood of depressed patients following treatment with escitalopram (31) and in hippocampus of rats subjected to early-life stress and then treated with fluoxetine, electroconvulsive shock therapy or ketamine (32).

To the best of our knowledge, thus far no studies have investigated the temporal effects of antidepressant treatments on the whole hippocampal miRNA expression profile. Therefore, main aim of the present work was to verify whether and how short-term treatments (for 3 and 7 days) with two different antidepressants, the tricyclic antidepressant desipramine and the selective serotonin reuptake inhibitor fluoxetine, could affect rat hippocampal miRNome.

Materials and methods

Animals and treatments

All animal procedures were conducted according to current regulations for animal experimentation in Italy (Decreto Legislativo 116/1992) and the European Union (European Communities Council Directive 2010/63/EU). A total of 54 male Sprague–Dawley rats were used;

27 for the 3 day- treatment and the same number for the 7 day- treatment (9 treated with fluoxetine, 9 with desipramine, both at 10 mg/kg/die, and 9 with water as vehicle by i.p. injection). All the animals were sacrificed at the same age. Each hemi-hippocampus from right or left hemisphere was taken separately and randomly assigned for RNA or protein analysis.

TaqMan array screen of antidepressants-induced microRNA expression

Total RNA including miRNA was extracted and purified by using mirVana™ miRNA Isolation Kit (Life Technologies Europe BV, Monza Italy). RNA quantity and quality were assessed by using a NanoVue plus spectrophotometer (GE Healthcare, version 4282 V1.7.3).

For each experimental group, RNA from 3 rats belonging to same group of treatment was pooled in equal amount to a final concentration of 1,5 ug/ul. 450 ng of total RNA were then retrotranscribed, according to the manufacturer protocol, using the TaqMan MicroRNA Reverse Transcription Kit and Megaplex™ RT Primers Sets A & B (Life Technologies). The cDNAs were then preamplified by using Megaplex PreAmp Primers Sets A & B (Life Technologies). Quantitative Real Time PCR (qRT-PCR) was performed by using TaqMan Array Rodent MicroRNA A+B Cards Set v3.0 on Applied Biosystem Fast 7900HT, following manufacturer's protocol (50°C for 2 min, 94,5°C for 10 min, followed by 40 cycles of 97°C for 30 sec and 59,7°C for 1 min). An equal number of control and treated samples were processed and assayed on the same day, by an individual who was unaware of group identity. Raw Ct values were extracted from filtered SDS files by using the Applied Biosystem SDS 2.3 software; Ct values were normalized by the $\Delta\Delta$Ct method on endogenous controls U6B, U87, Y1 and snoRNA135, whose mean value did not vary across groups. A total of about 400 miRNAs were detected in all samples (mean Ct value <35).

Bioinformatic analyses for the identification of miRNA putative target genes and functional annotation analysis

The putative target genes for each miRNA were determined by aggregating results from different prediction tools. We performed the analysis of the target site using the seed of the miRNA experimentally tested and searching them genome-wide against the dataset 3'-UTRs from the rat genome Rnor_5.0/ GCA_000001895.3, by employing different prediction tools: miRandav3.3a (33), TargetScanv5.1 [34], RNA-hybrid v2.0 (35) PicTar4way (36), DIANA-microT (37) and PITAv6 (38). This approach was necessary since the TaqMan® Array Rodent MicroRNA included, miRNAs of rat, mouse and human. To select predicted interactions, only 7–8 nucleotide matches were considered and only 1 G:U wobble base-pair and/or 1 mismatch was allowed. Results considered should be predicted by at least one algorithm plus PITA, with a negative binding energy.

The integration of the predictions was performed following a previous published approach of list re-ranking (39). The method has been further improved by using a Genetic Algorithm (GA) for the identification of the best miRNA target list, as implemented by the RankAggreg R package (40). In detail, the best miRNA target list is defined as the simultaneously closest as possible to the entire individual ordered lists provided by the different prediction algorithms, according to the Sperman footrule distance. The integrated lists were computed by applying a deep optimization through the GA (generation=30000, popsize=1000) provided by the RankAggreg package, in order to achieve the best target::miRNA predictions. The algorithm converged for the top 100 results, which were used for the following analyses. The lists re-ranking performed with the GA allows to score the final target::miRNA list (the fitness of the list according to the final iteration of the GS), providing an idea of the original consensus of the prediction obtained with the different algorithms.

In order to gain information about the biological networks regulated by the identified miRNAs, the target gene lists were characterized relying on functional annotation against the 3 domains of the Gene Ontology (cellular component, molecular function and biological process), computed using Biomart (41) and according to KEGG pathway terms (42).

The associations were classified by assigning a statistical significance relying on an enrichment analysis approach. The probability from hypergeometric distribution was computed for each annotation term (meaning both nodes and leaves). We assigned an estimate (p-value) to the hypothesis that a pool of selected target genes actually belongs to a specific annotation term more than what could be found simply by chance, following the model proposed by Tavazoie et al. (43). In detail, the total number of genes annotated with a specific GO term (tot) is used to calculate the expected value in the sample (exp), which is then statistically compared with the effective GO annotations observed in the sample (obs) to compute their enrichment and therefore their statistical significance in the sample.

Moreover, by employing semantic similarity we performed a clustering evaluation of the GO annotation terms. Cluster analysis was applied, relying on ontology similarity and performed through the GOSemSim package of R (44, 45). The Cluster silhouette measures were considered in order to describe how appropriately single observations are grouped together. Filtering out clusters of annotation terms characterized by negative silhouette values (probably heterogeneous) helped in further enriching the top of the target lists, keeping only targets annotated with the most relevant terms. Finally, in order to provide a graphical visualization of the clusters of GO terms identified, heatmaps were computed for the 3 branches of GO (Biological Process, Cellular Component and Molecular Function).

Selection and validation of genes/proteins as targets of antidepressants-regulated miRNAs

Considering that miRNAs could induce both mRNA degradation or inhibition of translation, we decided to analyzed by means of both Rt-PCR and western blot some of the putative target genes of miRNAs modulated by antidepressant treatments according to the following main criteria:

a) target of more than one miRNA significantly modulated by drug treatments; b) target of miRNA with FC greater than 3; c)target of miRNA similarly modulated by both desipramine and fluoxetine after 7 days of treatment.

Selected putative target genes and respective miRNAs are reported in Table S1.

RNA from each single sample was treated with the RQ1 RNase-free DNAse kit (Promega Italia S.r.l., Milano, Italy). cDNA-free RNA was then retrotranscribed by using the iScript cDNA Synthesis Kit (Bio-rad Laboratories S.r.l., Segrate, Milano, Italy). For the qRT-PCR analyses, RT2 qPCR Primer Assays (Qiagen S.r.l., Milano, Italy) for Adar2, Arid4a, Bcl6, Bmp7, Dnmt1, Galr1, Klf7, Kv4.2, Smad1, Smad2, Tgfbr1, Zip1, and β-actin and Rps18 as endogenous controls, were used. The RT2 qPCR Primer Assays were mixed with the RT2 SYBR Green/ROX qPCR Master Mix, and then dispensed into each well of a 96-well PCR Array with 10 ng of cDNA/well. The amplification protocol included 3 steps at 95 °C for 10 min for 1 cycle, then 95 °C for 15 s, 55 °C for 40 s, and 72 °C for 30 s, repeated for 40 cycles in an Applied Biosystem Fast 7900HT system.

Raw Ct values were extracted from filtered SDS files using the Applied Biosystem SDS 2.3 software; Ct values were normalized by the ΔΔCt method on endogenous controls, whose mean value did not vary across groups.

Preparation of subcellular fractions and Western blot analysis

Subcellular fractions (total homogenate, cytosol and nuclei) from emi-HPC (randomly right or left) from each animal were prepared as previously described (46, 47). Western blot analysis was carried out as previously described (46, 47), by incubating PVDF membranes, containing electrophoresed proteins from either nuclear or homogenate fractions, with antibodies against: Bcl6 (1:500), Smad2 (1:1000) and Dnmt1 (1:500) (all from Cell Signalling Technology Inc, Danvers, MA); Tgfbr1 (1:1000; Millipore S.p.A., Vimodrone, Italy); Bmp7 (1:500), Galr1 (1:1000), Klf7 (1:1000) and Smad1 (1:1000) (Abcam LTD, Cambridge, UK); Adar2 (1:1000) and Zip1 (1:1000) (Novus Biologicals LTD, Cambridge UK); Kv4.2 (1:1000; Alomone Labs LTD, Jerusalem, Israel). All protein bands used were within linear range of standard curves, and normalized for β-actin level (Sigma-Aldrich, 1:20:000) in the same membrane. Membranes were imaged using a LiCor Odyssey scanner and analyzed by using the Odyssey 3.1.4 analytical software (LiCor Biosciences, Lincoln, NE).

Statistical analysis

Statistical analysis for miRNA expression was performed by using SAM software (Significance Analysis of Microarrays, version 4.0, Stanford University, http://www-stat.stanford.edu/~tibs/SAM/), by setting the following parameters: two-class unpaired analysis, Student's t-test statistic, 5000 permutations and FDR <5%.

Student's T-test was also applied for statistical analysis for target validation studies by using out using Prism 5.0 (GraphPad Software Inc., USA).

RESULTS

Antidepressants induce an early and time-dependent modulation of hippocampal miRNome

Hippocampal miRNome expression profiles were analyzed in rats treated for 3 or 7 days with fluoxetine or desipramine, as described in the Methods section. The expression analysis of each antidepressant treatment group compared to the respective vehicle treated control group revealed a significant and specific effect of antidepressants on miRNA expression at the different time points. As shown in Figure 1, desipramine induced a significant increase in the expression of eight miRNAs after 3 days of treatment as well as of thirteen additional miRNAs after 7 days of treatment (Table S2). A quite different picture emerged from the analysis of the miRNome modulation after fluoxetine treatment. Indeed, we observed a significant downregulation of eight miRNAs after 3 days of treatment and significant changes in the expression of thirty-five miRNAs after 7 days of treatment, with the downregulation of 7 miRNAs and increased levels of twenty-eight miRNAs (Figure 1, Table S2).

Interestingly, after 7 days of treatment both antidepressants similarly modulated eight miRNAs, all upregulated (Table S2), thus suggesting the presence of common miRNA targets for fluoxetine and desipramine. On the other hand, no one of the miRNAs modulated by one of the two drugs after 3 days of treatment was significantly modified after 7 days of treatment.

Computational analysis of miRNA targets and pathways

miRNA Target Prediction

A single miRNA can target hundreds of mRNA transcripts and computational prediction of miRNA targets is a critical initial step in identifying miRNA:mRNA target interactions for

experimental validation. To this aim, we used a specifically designed computational approach based on a previously described method (39). Potential target genes of the miRNAs significantly modulated by antidepressant treatments were identified by using six different prediction tools (see Methods). The top 100 targets for each miRNA are shown in Table S3. As it could be expected, several of the putative target genes, such as 5HT2B receptor, several glutamatergic receptors (i.e. AMPA, NMDA, kainate, and metabotropic receptors), D1A, D2, A2R, CB1 and CB2, COMT, PKC, CaMKIV, MapK, Trkb, phospholipase C and D, CREM, c-fos, GAD1, Munc-18, TGFbR1, wnt9, Map1b, wnt, Vgf, Bcl2; mTOR, and different synaptic proteins (SNAP25, Syt1, Syt2, Stx1a, Vamp) among the others, are already known for their involvement in the mechanism of action of antidepressants and/or in the pathophysiology of mood and anxiety disorders.

Interestingly, some of the genes identified are putative targets of different miRNAs (Table S4). The greater number of common targets for at least 3 different miRNAs was found for the miRNAs significantly modulated by 7 days of fluoxetine treatment (n = 143; Table S4), also consistent with the higher number of miRNAs regulated (35) at this time point.

Regarding the other groups, namely 3 and 7-day treatments with desipramine and 3 days with fluoxetine, a mean of ten different genes were found to be putative targets of at least 3 different miRNAs, as reported in Table S4.

Functional Annotation and Enrichment Analysis

In order to identify the molecular and functional annotations and canonical biological pathways potentially influenced by target genes of differentially expressed

miRNAs after treatment with desipramine or fluoxetine, we performed an annotation analysis with the Biological Process, Cellular Component, and Molecular Function domains of Gene Ontology (GO) and the KEGG pathways following an enrichment analysis approach (41, 42). Table 1 reports the 15 most enriched and significant pathways in the GO Biological Process domain for 3 and 7 day-treatments with desipramine, whereas Table 2 reports the data relative to fluoxetine treatments. The complete data regarding the whole overexpressed Biological process terms, the other GO domains (Cellular component and Molecular function) as well as the KEGG pathways can be found in the Supplementary materials (Table S5). The ranking of the different pathways in the tables was based on the higher number of genes observed in each pathway and on the p value.

As highlighted by these lists, for both drugs, an enrichment in pathways associated with a variety of cellular processes is detectable after 3 days of treatment. In details, regarding desipramine (Table 1), on a total of 46 pathways significantly enriched, the first term is Intracellular signal transduction, (15 putative target genes of miRNAs significantly modulated; p= 0,011), followed by DNA repair (9 putative targets; p= 0,017), and Calcium ion transport (7 putative targets, p=0,047). Additional terms directly linked to the central nervous system, such as Neuron projection development and Axonogenesis, are also present.

We then performed a further analysis in order to highlight the presence of significant clusters among the overexpressed GO annotation terms for 3 days of desipramine treatment. With regard to the Biological process category, as highlighted by the heat map (Figure 2a), we found one significant cluster including the following terms: DNA recombination, double-strand break repair via homologous recombination, postreplication repair, DNA repair and pyrimidine dimer repair, suggesting a significant involvement of genes linked to DNA modulation. Genes included in the pathways are different types of DNA polymerases (eta, theta, and kappa),

ubiquitin-conjugating enzymes (i.e., Ube2b, Ube2n), and asf1a and Morf4l1. The last two are involved in epigenetic mechanisms, being a key histone H3-H4 chaperone and an histone acetyltransferase, respectively. The heat maps highlighting the cluster analyses results related to the other GO domains (Cellular component and Molecular function) are reported in the Supplementary section (Figure S1 a and b).

After 7 days of treatment with desipramine we found that as many as 97 pathways in the GO Biological Process domain were significantly enriched in miRNA putative target genes (Table S5). As shown in Table 1, the first 3 pathways are In utero embryonic development (23 putative targets; p= 0.032), Kidney development (12 putative targets; p= 0.034) and Cellular calcium ion homeostasis (11 putative targets; p=0.005). Although the first two pathways may seem unrelated to CNS, they contain several genes expressed in the brain, such as Pdgfrb, Tgfbr1, Smad1, Bmp7, Bmpr1a, Wnt9b. Several among the overexpressed terms are associated to neurotransmission, as shown also from the cluster analysis, in which 3 main clusters were found (Figure 2b). The first one is related to transport of lipids (cholesterol efflux, lipid transport and sterol transport); the second and richest in number of terms is related to neurotransmission (including pathways such as regulation of neurotransmitter secretion, long-term synaptic potentiation, regulation of GABAergic synaptic transmission and regulation of glutamatergic synaptic transmission), and the third is related to signaling pathways such as MAPK and mTor. Significant clusters were also identified among the GO molecular function terms and Cellular components terms (Figure S1c and S1d).

Regarding fluoxetine, after 3 days of treatment we found a significant enrichment in miRNA target genes in 52 pathways in the biological process domain (Table S5). According to the previously described criteria, as shown in Table 2, the first 3 pathways are In utero embryonic development (15 putative targets; p=0.0384), Post-embryonic development (9 putative targets;

p=0.0042), and Response to DNA damage stimulus (9 putative targets; p=0.0153). As illustrated above for desipramine, these pathways contain many genes expressed in CNS, as for some of the additional pathways clearly related to CNS, such as Behavior, Long term synaptic depression, Dentate gyrus development, Hippo signaling cascade, Brain segmentation, Dopamine transport, Adrenergic receptor signaling pathway, Beta-amyloid metabolic process, Cerebral cortex GABAergic interneuron migration and Long-term synaptic potentiation.

As highlighted by the related heat map (Figure 2c), the cluster analysis showed the presence of 3 main clusters in the biological process category, the first one related to tRNA aminoacylation, the second one to embryonic development, and the third one to differentiation of lymphocyte and osteoclasts.

A significant cluster was also found in the Molecular function GO terms whereas no one was found in the cellular component terms (see Figure S2a).

A greater enrichment of putative miRNA target genes was found for the 7 days treatment with fluoxetine, where 71 pathways (Table S5) were identified. As shown in Table 2, among the top Biological process terms, we found Cell adhesion (70 putative targets; p=0,0112), embryonic development (48 putative targets; p=0,0037) and synaptic transmission (25 putative targets, p=0.0142). Other pathways such as Sodium ion transport, Neuron differentiation, Axonogenesis, Regulation of neuronal synaptic plasticity and Brain morphogenesis were also found to be significantly enriched. Moreover, various pathways related to cholesterol and triglycerides synthesis/transport are present. This kind of enrichment was also confirmed by the cluster analysis (Fig 2d), that revealed four main clusters: the first one comprising synaptic transmission, rhythmic excitation and regulation of neuronal plasticity. The second was related to morphogenesis of organs including brain and to pituitary gland development; the third

comprising cell morphogenesis involved in differentiation, Schwan cell differentiation, neuron differentiation, axonogenesis, and dendrite morphogenesis and, finally, the last one related to cholesterol/sterol transport. As shown in Figure S2 (b and c), significant clusters were also found in the overexpressed molecular function and cellular component terms.

miRNAs modulated by both drug treatments. Analysis of target genes

Considering that eight miRNAs were similarly modulated by both desipramine and fluoxetine after 7 days of treatment (Table S2), a bioinformatic analysis similar to that described above, was performed on these specific miRNAs in order to identify possible common targets. In this case, we found a significant enrichment of miRNA putative target genes in 59 pathways belonging to the Biological process domain of GO, 22 pathways belonging to the Cellular components, 31 to Molecular function and 8 KEGG enriched pathways (Tab S5). Regarding the Biological process enriched pathways, the first 3 terms are In utero embryonic development (17 putative targets; $p=0.019$), intracellular signal transduction (16 putative targets; $p=0.042$) and Anti-apoptosis (13 putative targets; $p=0.026$). Interestingly, a great number of the remaining pathways are related to the CNS, such as MAPK cascade, cell-cell signaling, regulation of neurotransmitter secretion, synaptic vesicle exocytosis, sleep, dendrite morphogenesis, among the others (Table S5). As shown in Figure 2e, the heat map highlighted the presence of 3 significant clusters among the overexpressed terms in the Biological process category. The first one is related to morphogenesis, and includes skin morphogenesis and heart morphogenesis among the others. The second one encompasses elements mainly related to neurotransmission, such as positive regulation of neurotransmitter secretion, regulation of neurotransmitter secretion, synaptic vesicle exocytosis, synaptic vesicle docking involved in

exocytosis, regulation of calcium ion-dependent exocytosis and regulation of endocytosis. Finally, the third one comprises different signaling pathways: regulation of smoothened signaling pathway, regulation of TOR signaling cascade, MAPK cascade, steroid hormone receptor signaling pathway and androgen receptor signaling pathway. Significant clusters were also identified among overexpressed molecular function and Cellular components terms (Figure S3).

Validation of miRNA putative target genes

Several interesting putative target genes of miRNAs modulated by antidepressant treatments emerged from the bioinformatic analysis. Based on the criteria described in the Methods section we analyzed the mRNA and protein expression levels of several putative target genes of miRNAs significantly modulated by antidepressants after three or seven days of treatment (Table S1).

Regarding the 3 day-treatment (Table 3), we analyzed the expression of several putative targets of miR-291a-3p (markedly downregulated after fluoxetine treatment). We found a significant increase in mRNA expression levels of Galanin receptor 1 (GalR1) with fluoxetine, without significant changes in protein levels. Instead, we found that the mRNA, but not protein, expression levels of Bcl6, another putative target of miR-291a-3p, was significantly reduced after treatment with fluoxetine. Also, both mRNA and protein expression levels of DNMT1 (DNA (cytosine-5)-methyltransferase 1), were not modified by fluoxetine treatment.

Moreover, in line with miR-294 increase after desipramine treatment, we observed a marked and significant dowregulation of the protein expression level of Smad2 (a key component of the Tgfb signaling pathway), with no significant changes in its mRNA levels. Smad2 is also a putative target of miR-291a-3p, but contrary to expectations its protein expression was

significantly reduced after treatment with fluoxetine. No modifications in mRNA or protein expression levels were found for the Tgfb receptor 1 (TgfbR1), a putative target of miR-182, downregulated by fluoxetine.

Regarding the 7 day-treatment (Table 3), the expression analysis highlighted a marked increase in protein levels of Adar2 (adenosine deaminase, RNA-specific, B1) a key enzyme in editing processes of RNAs including miRNAs, after desipramine but not fluoxetine treatment, without any changes in its mRNA levels. Adar2 is a putative target of two miRNAs similarly modulated by both desipramine and fluoxetine (miR-129-5p and miR-202-3p) as well as of miR-7b and miR342-3p, modulated by fluoxetine.

BMP7 (bone morphogenetic protein 7), which belongs to the Tgfb signaling superfamily, was modified by both desipramine and fluoxetine treatment; indeed we found a marked and significant reduction of its protein expression following desipramine and a trend toward a significant reduction after fluoxetine treatment, without any significant change in mRNA levels. The reduction observed after desipramine treatment was in line with increased expression of miR-712. The expression of Galr1, putative target of miR-1a-3p (increased by fluoxetine), different from the result after three days of treatment, was not modified after seven days of fluoxetine. The protein expression levels of the transcription factor Klf7 (Kruppel-Like Factor 7), putative target of miR-1941 and miR-202-3p, both upregulated by desipramine treatment, and of miR-125a-3p (upregulated by fluoxetine) was significantly increased after desipramine, but not fluoxetine treatment.

As many as 5 miRNAs upregulated by desipramine and 4 modulated by fluoxetine (3 upregulated and one reduced) were predicted to target the potassium channel Kv4.2. In line with this prediction, we observed a significant reduction in its protein expression levels in

hippocampus of rats treated with desipramine. Instead, no significant change was found in fluoxetine-treated animals. miR-129-5p, that was similarly upregulated by both desipramine and fluoxetine treatment, was predicted to regulate, among the other targets, Smad1, another main component of the Tgfb signaling pathway. Again, contrary to expectations, the expression analysis revealed a significant increase in Smad1 protein levels following desipramine, but not fluoxetine treatment. TgfbR1 is a putative target of miR-1982.2 (significantly increased by both desipramine and fluoxetine) and of other miRNAs modulated by fluoxetine after seven days of treatment (miR-205, miR-342-3p and miR-509). In line with prediction, we observed a significant downregulation of TgfbR1 mRNA after desipramine treatment and a trend toward a reduction after fluoxetine treatment. No significant changes were observed in the protein expression levels after treatment with the two drugs.

Finally, we measured the expression levels of Zip1 (SLC39A1), a member of the Zip membrane Zn transporter family, predominantly expressed in the hippocampus, that was predicted as putative targets of four miRNAs similarly modulated by desipramine and fluoxetine. However, as shown in Table 3, no significant changes were found in both mRNA and protein expression levels.

Overall, we found that five genes, namely Bmp7, Galr1, Kv4.2, Smad2 and Tgfbr1, on a total of 12 analyzed, showed changes in their expression in line with those observed in miRNAs; the expression of four genes was modified in the opposite direction to what expected and for two target genes we did not find any significant modification.

Discussion

Main aim of this study was to investigate whether miRNAs may be effectors of antidepressant treatment at different time lengths. The present results showed for the first time that

desipramine and fluoxetine induce an early and time-dependent modulation of hippocampal miRNome expression profile. Interestingly, the two antidepressants exert different effects on miRNAs expression at the different time points assessed. In particular, after three days of treatment a significant upregulation of eight miRNAs was found after treatment with desipramine whereas fluoxetine decreased the expression of eight different miRNAs (Figure 1). After seven days of treatment, while desipramine induced a significant upregulation of thirteen miRNAs, fluoxetine modified the expression of thirty-five miRNAs, upregulating twenty-eight and reducing seven miRNAs (Figure 1). Intriguingly, the expression of eight miRNAs was similarly increased by both drugs after seven days of treatment, thus suggesting that these miRNAs, as well as their targets, could be regulated in a similar way by the administration of two structurally and functionally different drugs.

A number of miRNAs we found significantly modulated by drug treatments have been previously shown to be involved in the pharmacotherapy of mood disorders. In particular, in line with our data showing an increased expression of miR-15 after 7 day-treatment with fluoxetine, an upregulation of miR-15 was reported in lymphoblastoid cell lines from bipolar patients after lithium treatment (48). Mir-21, found to be reduced here by 7 days of desipramine, was also reduced in human lymphoblastoid cell lines after paroxetine treatment (49). Interestingly, miR-129-5p, upregulated by 7 days of treatment with both desipramine and fluoxetine and miR-205-5p, upregulated by seven days of fluoxetine treatment, were previously shown to be increased after repeated electroconvulsive shock treatment (32).

Other miRNAs significantly modulated by fluoxetine and desipramine, were also reported to be involved in the pathophysiology of mood disorders. A single nucleotide polymorphism in the precursor of miR-182, causing the overexpression of the mature form of this miRNA (that we found to be downregulated by 3 days of fluoxetine treatment), has been associated with late

insomnia in depressed patients (50). miR-488, upregulated by seven days of treatment with both fluoxetine and desipramine, was found to be involved in physiological pathways linked to the development of anxiety disorders, and in particular of panic disorder (51). Finally, we have found that miR-511, previously shown to be reduced in prefrontal cortex of depressed suicide subjects (52), was upregulated after seven days of treatment with both fluoxetine and desipramine.

Bioinformatic analysis: putative target genes and pathways involved

The bioinformatic analyses performed in order to identify putative target genes of miRNAs significantly modulated by desipramine and fluoxetine treatments produced lists of hundreds of genes. Among the putative miRNA target genes, several are known for their involvement in the mechanism of action of antidepressants and/or in the pathophysiology of mood and anxiety disorders (i.e., 5HT2B receptor, several glutamatergic receptors, D1A, D2, a2R, CB1 and CB2, PKC, CamKIV, MapK, Trkb, phospholipase C and D, CREM, c-fos, GAD1, Munc-18, TGFbR1, Map1b; mTOR, and different synaptic proteins; Table S4) (2, 53-57). The annotation analysis showed for both drugs, an enrichment in pathways associated with a great variety of cellular processes. In particular, pathways related to neuronal plasticity (i.e., regulation of synaptic transmission, LTP, LTD, synaptic vesicle docking, MAPK cascade, among the others; Table S4) emerged at both times of treatment, particularly when considering the eight miRNAs similarly modulated by the two drugs after seven days of treatment. Epigenetic mechanisms also seem to be involved, due to the presence of different pathways related to DNA/RNA regulation (i.e., Histone acetyltransferase, DNA repair, methylated histone residue binding, DNA-directed DNA polymerase activity; Table S4), especially after three days of desipramine treatment.

Putative target genes analysis

We analyzed the mRNA and protein expression levels of selected putative target genes (Table S2) of miRNAs significantly modulated by the two drugs after three or seven days. Our results showed, for some targets, a modulation in line with miRNA expression levels. In particular, regarding three days of treatment, we found a significant upregulation in mRNA levels of Galr1, a putative target of mir-291a-3p (strongly downregulated by 3 days of fluoxetine treatment. Galanin is a predominantly inhibitory neuropeptide that is co-expressed with and modulates noradrenaline and serotonin transmission. Several studies have suggested that the galanin pathway could be involved in various human diseases and pathological conditions, including seizures, Alzheimer's disease, mood disorders, anxiety and stress response (58, 59). A significant downregulation, in line with miR-294 increase after three days of desipramine treatment, was found for Smad2, a key component of TGFb signaling pathway, which in conjunction with Smad4, can regulate transcriptional responses in the nucleus, both positively and negatively (60). Significant modulation in the expression levels of two other key components of Tgfb signaling pathway, namely Bmp7 and Tgfbr1, were also found after seven days of antidepressant treatments. Indeed, protein expression levels of Bmp7 were significantly reduced, consistent with miR-712 upregulation after desipramine treatment. BMPs are retrograde, trans-synaptic signals that could affect presynaptic growth and neurotransmission, and seem to a have a key role in hippocampal development and neurogenesis (61, 62). Also Tgfbr1 mRNA expression levels were reduced after desipramine treatment. This receptor (putative target of several miRNAs, including miR-1982.2, that was similarly upregulated by both desipramine and fluoxetine) is a key component of TGFb family, a pathway with well-established roles in neurotrophic and neuroprotective mechanisms (54). Finally, after seven days of desipramine treatment, we found a significant decrease in protein

expression levels of the potassium channel Kv4.2. This gene is a putative target of several miRNAs, and in particular of five miRNAs upregulated by desipramine (Table S1). This channel, which plays a crucial role in controlling neuronal excitability by mediating transient A-type potassium currents, has been directly associated with spatial memory in rats and has been implicated in a number of hyperexcitability-related and neurodegenerative diseases (63). Moreover, knockout mice for this gene have shown an increase in LTP in hippocampal CA1 pyramidal neurons (64).

Interestingly, other genes (Smad 2 after three days of fluoxetine treatment and Adar2, Klf7 and Smad 1 after seven days of desipramine treatment) showed a positive correlation with miRNAs expression levels, instead of the inverse correlation that would be expected if the miRNAs simply inhibited translation of their gene targets. These findings could be explained by different factors: miRNAs appear to be co-regulated with their targets, a phenomenon that could be explained considering the miRNA – mRNA interactions constitute a complex network. Recent studies have indeed suggested that miRNAs may act not only by merely reducing protein translation, but also through non conventional mechanisms (i.e. leading to increase in protein expression levels, binding target genes at 5'UTR region, guiding transcription factors to target promoter) (14, 15).

In conclusion, this work has shown that miRNAs may contribute to the antidepressant mode of action through different patterns. The different modes of gene expression regulation involving miRNAs suggest that additional work is needed in order to fully understand their exact role and to develop new avenues for therapeutic strategies.

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Figures and tables

Figure 1. Time dependent modulation of hippocampal miRNA expression by desipramine or fluoxetine treatment for three or seven days

Graphical representation of miRNAs significantly modulated in hippocampus of rats after treatment with desipramine (a) or fluoxetine (b) for 3 or 7 days. Data are expressed as fold changes versus vehicle treated rats. SAM analysis ($p < 0.05$, FRD<5%).

Figure 2. Hierarchical clustering of enriched annotations in the GO Biological process category for predicted targets of miRNAs modulated by antidepressant treatments.

The heat maps highlight significant clusters of the GO-Biological process terms enriched in putative target genes of miRNAs modulated by: (a) 3 day-treatment desipramine; (b) 7 day-treatment desipramine; (c) 3 day-treatment fluoxetine; (d) 7 day-treatment fluoxetine; (e) 7-day treatment, common to desipramine and fluoxetine. The color ramp of the heatmap is defined according to the values of similarity score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1). Green squares highlight significant clusters (described in the text).

Table 1. Significantly overexpressed terms in the GO – Biological Process category and enriched genes for miRNAs modulated by desipramine treatment.

Table reports the first 15 significantly enriched terms ranked on the number of observed genes and p value, after three and seven days of desipramine treatment.

Tot = the total number of genes annotated with a specific GO term; Exp = the expected value of genes in the sample (exp); Obs = effective GO annotations observed in the sample. Statistics: Fisher's exact test.

Table 2. Significantly overexpressed terms in the GO – Biological Process category and enriched genes for miRNAs modulated by fluoxetine treatment.

Table reports the first 15 significantly enriched terms ranked on the number of observed genes and p value, after three and seven days of fluoxetine treatment.

Tot = the total number of genes annotated with a specific GO term; Exp = the expected value of genes in the sample (exp); Obs = effective GO annotations observed in the sample. Statistics: Fisher's exact test.

Table 3. Expression analysis of putative target genes of miRNAs modulate after treatment with desipramine or fluoxetine for three or seven days.

Table reports the expression analysis of mRNA and protein levels of putative target genes of miRNAs modulated by desipramine and fluoxetine treatment.

Data on mRNA levels are expressed as fold changes (FC) vs vehicle treated rats; data on protein levels are expressed as % vs vehicle treated rats. In bold, significant changes vs vehicle treated animals. Statistics: Student' t test.

Figure S1. Hierarchical clustering of enriched annotations in the GO Cellular Component and Molecular Function category for predicted targets for miRNAs modulated by desipramine treatments.

The heat maps highlight significant clusters of the Cellular Component and Molecular Functions GO terms enriched in putative target genes of miRNAs modulated by desipramine: (a) 3 day-treatment, Cellular component; the terms included in the significant cluster (green square) are Apical part of cell, Plasma membrane and Intrinsic to membrane; (b) 3 day-treatment, Molecular Function; the terms included in the significant cluster (green square) are Cytokine activity, Interleukin-1 receptor binding and Epidermal growth factor receptor binding; (c) 7 day-treatment, Cellular component; the terms included in the significant cluster (green square) are Synaptosome, Microsome and Vesicular Fraction ; (d) 7 day-treatment, Molecular Function; 3 significant clusters are highlighted by green squares. The terms included in the first cluster are 5'-nucleotidase activity, Inositol- or phosphatidylinositol- phosphatase activity and Inositol-polyphosphate 5- phosphatase activity; the terms included in the second cluster are Adenylylsulfate kinase activity, Phosphotransferase activity alcohol group as acceptor, Inositol- or phosphatidylinositol- kinase activity and phosphatidylinositol- 4,5- biphosphate 3-kinase activity. The terms included in the third cluster are CARD domain binding, Kinase binding, Tubulin binding, SMAD binding, Histone binding, Wnt- protein binding, SNARE binding and protein binding. The color ramp of the heatmap is defined according to the values of similarity

score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1).

Figure S2. Hierarchical clustering of enriched annotations in the GO Cellular Component and Molecular Function category for predicted targets for miRNAs modulated by fluoxetine treatments.

The heat maps highlight significant clusters of the Cellular Component and Molecular Function GO terms enriched in putative target genes of miRNAs modulated by fluoxetine: (a) 3 day-treatment, Molecular Function; the terms included in the significant cluster (green square) are GTPase binding, RNA npolymerase binding, RS domain binding, Semaphorin receptor binding, GTP-dependent protein binding and S100 alpha binding; no significant clusters were found in the Cellular Component terms; (b) 7 day-treatment, Cellular component; 2 significant clusters are highlighted by green squares. The terms included in the first cluster are Cytoplasmic vesicle, Late endosome and Golgi apparatus; the terms included in the second cluster are Plasma membrane, Apicolateral plasma membrane and Lateral plasma membrane; (d) 7 day-treatment Molecular Function; the terms included in the significant cluster (green square) are Actin binding, Cytoskeletal protein binding, ATP-dependent protein binding and Phosphoprotein binding. The color ramp of the heatmap is defined according to the values of similarity score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1).

Figure S3. Hierarchical clustering of enriched annotations in the GO Cellular Component and Molecular Function category for predicted targets for miRNAs similarly modulated by seven days of desipramine and fluoxetine treatments.

The heat maps highlight significant clusters of the Cellular Component and Molecular Function GO terms enriched in putative target genes of miRNAs similarly modulated by seven days of desipramine and fluoxetine: (a) Cellular Component; 2 significant clusters are highlighted by green squares. The terms included in the first cluster are Guanyl-nucleotide exchange factor complex, Phosphopyruvate hydratase complex and Phosphatidylinositol 3-kinase complex; the terms included in the second cluster are Nuclear body, Histone deacetylase complex, Mediator complex and Transcriptional repressor complex; (b) Molecular Function; 2 significant clusters are highlighted by green squares. The terms included in the first cluster are Protein kinase C binding, Kinase binding, CARD domain binding, Fibroblast growth factor binding, Tropomyosin binding, ATP-dependent protein binding, SNARE binding and Histone binding. The terms included in the second cluster are Inositol or phosphatodylinositol kinase activity, 1-phosphatidylinositol- 3-kinase activity and phosphatidylinositol- 4,5- biphosphate 3- kinase activity. The color ramp of the heatmap is defined according to the values of similarity score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1).

Table S1. Putative target genes of miRNAs significantly modulated by antidepressant treatments assessed for validation study

Table reports the putative target genes and respective miRNAs selected for validation studies according to the following criteria:

a) target of more than one miRNA significantly modulated by drug treatments; b) target of miRNA with FC greater than 3; c) target of miRNA similarly modulated by both desipramine and fluoxetine after 7 days of treatment.

Table S2. miRNAs significantly modulated by desipramine or fluoxetine treatments

Fold changes and q values for miRNAs significantly modulated by desipramine or fluoxetine treatments (SAM analysis, FDR<5%, p<0.05).

Q Value= the lowest False Discovery Rate at which that gene is called significant.

In bold, miRNAs similarly modulated by desipramine and fluoxetine treatment for seven days.

Table S3. Putative target genes of miRNAs modulated by antidepressant treatments.

Table reports the first 100 best targets predicted for each miRNA significantly modulated by three days of desipramine (DMI 03), seven days of desipramine (DMI 07), three days of fluoxetine (FLX 03), seven days of fluoxetine (FLX 07) treatment.

In blu the score for each target::miRNA list (the fitness of the list according to the final iteration of the GS), that provides an idea of the original consensus of the prediction obtained with the different algorithms.

When present, at the end of each list are highlighted in red target genes experimentally validated elsewhere.

Table S4. Putative target genes modulated by more than three miRNAs.

For each drug treatment are listed putative target genes of more than three miRNAs

Table S5. Significantly overexpressed terms and enriched genes in the GO –categories and KEGG pathways for miRNAs modulated by antidepressant treatments

The file reports the overexpressed terms in the GO biological process, Molecular function, cellular components and KEGG pathways, together with the respective enriched genes for miRNAs modulated by:

a) Three days of desipramine treatment (DMI 03); b) seven days of desipramine treatment (DMI 07); c) three days of fluoxetine treatment (FLX 03); d) seven days of fluoxetine treatment (FLX 07); e) miRNAs commonly modulated by seven days of desipramine and fluoxetine treatments (DMI-FLX 07).

Enriched terms in are ranked on the number of observed genes and p value. Tot = the total number of genes annotated with a specific GO term; Exp = the expected value of genes in the sample (exp); Obs = effective GO annotations observed in the sample. Statistics: Fisher's exact test.

Enriched genes are listed according to the alphabetical order of the term to which they belong.
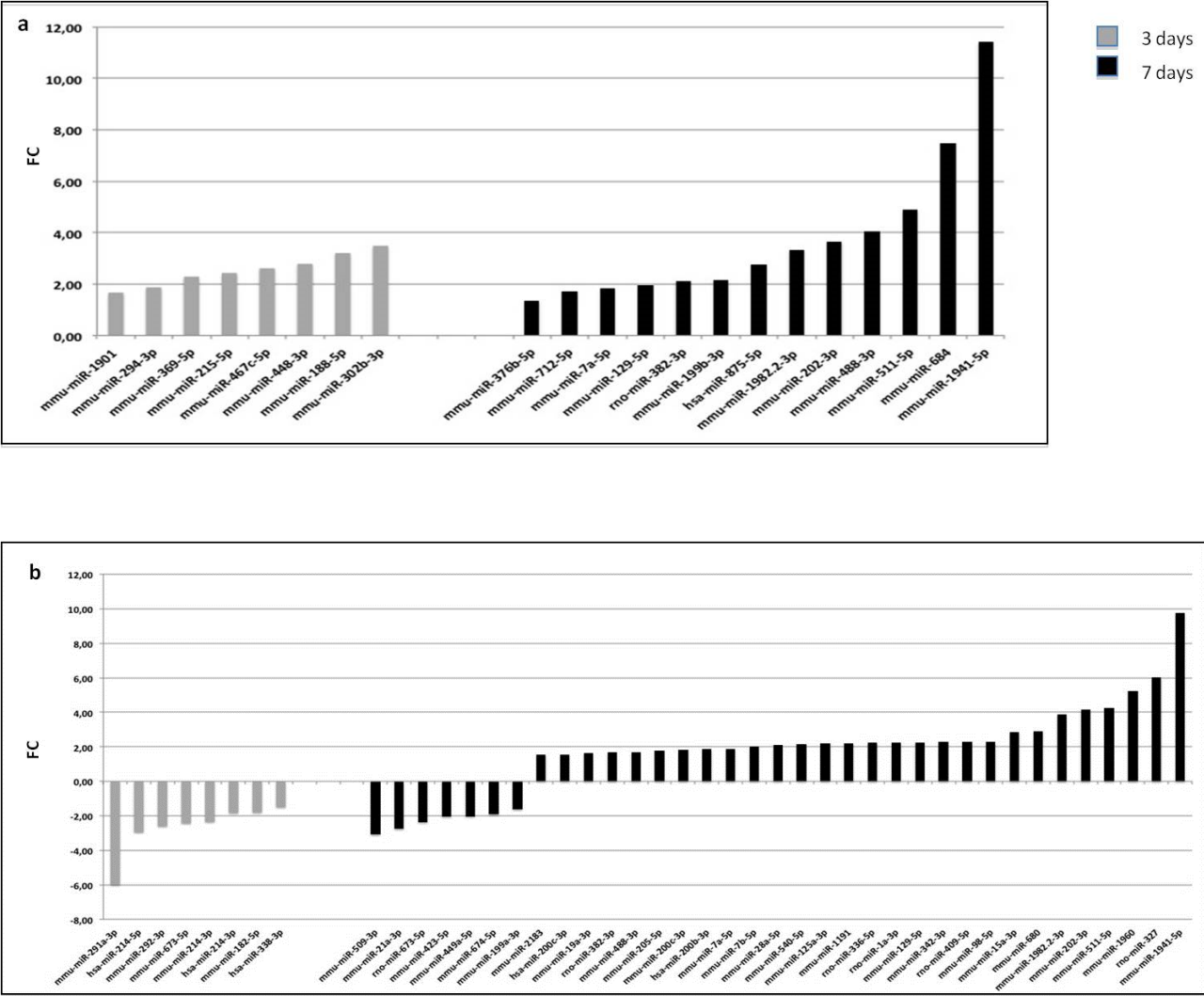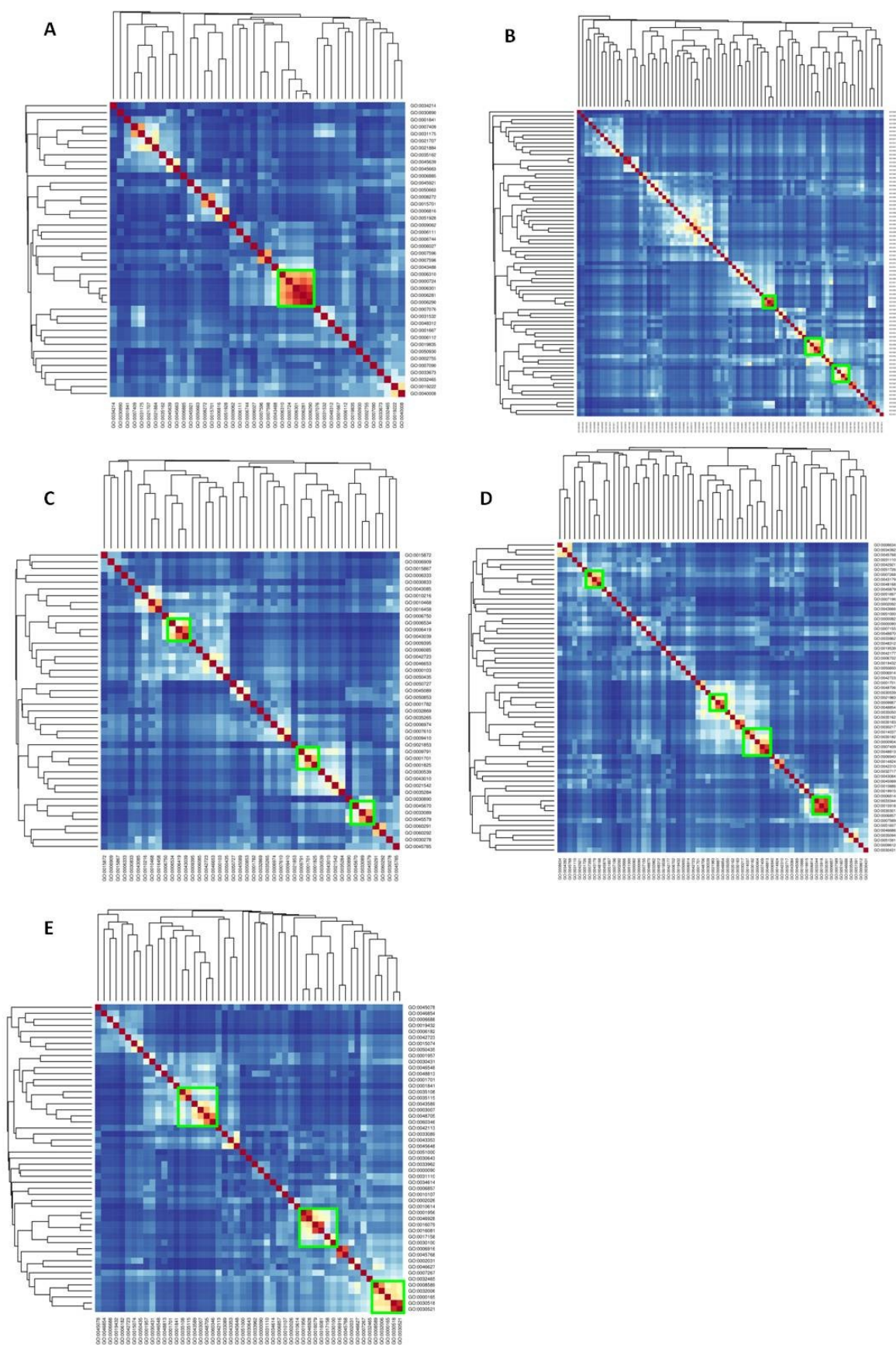
Figure 1

Figure 2

Table 1: Significantly overexpressed terms in the GO – Biological Process category and enriched genes for miRNAs modulated by desipramine treatments.

Desipramine treatment for three days					
Term and Description	**Tot**	**Exp**	**Obs**	**P value**	**Enriched putative target genes**
GO:0035556 - Intracellular signal transduction	120	8	15	0,0115	Tesk2, Pkca, Pkc, Myo9b, Lep, Wsb2, Cnih, Dcdc2, Smad2, RGD1308448, Socs5, Lnk, Grb14, Shc1
GO:0006281 - DNA repair	61	4	9	0,0171	Ube2n, Chek1, Polk, Polq, Giyd2, Asf1a, Morf4l1, Ercc6, Ube2b
GO:0006816 - Calcium ion transport	51	3	7	0,0473	Atp2c1, Atp2b2, Cacnb3, Cacna1g, Panx1, Slc24a6, Slc24a2
GO:0007596 - Blood coagulation	32	2	6	0,0162	F2r, F31, Ifnar1, Tfpi, Ifngr2, F13a
GO:0002755 - Myd88-dependent toll-like receptor signaling pathway	34	2	6	0,0216	Ube2n, Irak4, Peli1, Map3k7, Ticam2, Mapk14
GO:0031175 - Neuron projection development	36	2	6	0,0280	Axcam, Rb1, Hmg1, Igf1r, Prickle2
GO:0007409 - Axonogenesis	39	3	6	0,0398	Cnp, Igfr1, Map1b, Ntng1, Uchl1, Slitrk5
GO:0006885 - Regulation of pH	12	1	4	0,0059	Slc9a2, Slc26a4, Slc9a8, Ednra2
GO:0030890 - Positive regulation of B cell proliferation	18	1	4	0,0268	Il7, Irs2, Vav3, Peli1
GO:0006310 - DNA recombination	19	1	4	0,0322	Maf2, Recql5, Giyd1, Morf4l1, Exo1
GO:0040008 - Regulation of growth	20	1	4	0,0383	Morf4l1, Igsf11, Nell2, Shc1
GO:0006301 - Postreplication repair	3	0	3	0,0003	Ube2n, Polh, Ube2b
GO:0006744 - Ubiquinone biosynthetic process	7	0	3	0,0081	Coq3, Pdss2, Coq9
GO:0035162 - Embryonic hemopoiesis	8	1	3	0,0123	Klf1, Myst3, Lnk
GO:0050930 - Induction of positive chemotaxis	9	1	3	0,0175	Pkca, Cxcl12, Hmg1
Desipramine treatment for seven days					
Term and Description	**Tot**	**Exp**	**Obs**	**P value**	**Enriched putative target genes**
GO:0001701 – In utero embryonic development	118	16	23	0,0320	Cited2, Eno1, Gad67, Gja1, Pdgfrb, Tpm1, Foxa2, Man2a1,

					Tgfbr1, Cul3, Sox8, Wnt9b, Tctn1, Ell, Rpgrip1l, Dnmt3l, Ncor2, Rai17, Zmiz1, Xrcc2, Ybx1, Tmed2, Bmpr1a, Cdh1
GO:0001822 - Kidney development	52	7	12	0,0340	Six1, Sulf1, Znf354a, Pdgfrb, Wt1, Smad1, Dcn, Tgfbr1, Wnt9b, Rpgrip1l, Lrp4, Bmp7
GO:0006874 - Cellular calcium ion homeostasis	36	5	11	0,0050	Atp2c1, Pgm1l, Pkcb, Cav1, Gcm2, Slc37a4, Atp2a2, Sypl2, Cacnb4, Vroac, Slc24a2
GO:0000165 - MAPKKK cascade	27	4	10	0,0015	Adra2b, Nf1, Raf1, Cav1, Smad1, Pbp, Ppm1l, Map3k7, Itpkb
GO:0006200 - ATP catabolic process	42	6	10	0,0417	Abcg5, Abcg8, Abcb12, Atp1a1, Atp1a2, Ide, Hltf, Atp2a2, Rhobtb3, ABCR
GO:0007267 - Cell-cell signaling	30	4	9	0,0122	Cx43, Sdc4, Cd24, Bhlha15, Panx2, Cx26, Cx40, Fzd1, Fgfr3
GO:0016311 - Dephosphorylation	32	4	9	0,0188	Atp1a1, Mdp-1, Ppp1r15b, Thtpa, Ppm1l, Inpp5d, Nt5e, Bpnt1, Synj2
GO:0090090 - Negative regulation of canonical Wnt receptor signaling pathway	37	5	9	0,0460	Siah2, Cav1, Fzd6, Dkk2, Scyl2, Rilp2, Fzd1, Lrp4, Sfrp4
GO:0045768 - Positive regulation of anti-apoptosis	18	2	8	0,0011	Dusp1, Il6r, Pdgfrb, Cav1, Star, Smad1, Lrp, Hsp60
GO:0006006 - Glucose metabolic process	27	4	8	0,0192	Pik3ca, Pgm1l, Hnf6, Crem, H6pd, Inppl1, Serp1, Kir6.2
GO:0070588 - Calcium ion transmembrane transport	31	4	8	0,0425	Grin3a, Htr2b, Serca2, Gpm6a, Cacnb4, Trpv4, Asic1, Slc24a2
GO:0001756 - Somitogenesis	21	3	7	0,0144	T, Sema3c, Myf5, Lrp6, Tbx18, Tmed2, Bmpr1a
GO:0051781 - Positive regulation of cell division	20	3	6	0,0385	Vegfc, Tgfa, Ptn, Htr2b, Tal1, Ybx1
GO:0000082 - G1/S transition of mitotic cell cycle	21	3	6	0,0481	Eif4e, Cdc25a, Spdya, Rb1, E2f1, Mtor
GO:0006869 - Lipid transport	21	3	6	0,0481	Star, Apoc2, Sigmar1, Lrp10, Gulp1, Apold1

Table 2. Significantly overexpressed terms in the GO – Biological Process category and enriched genes for miRNAs modulated by fluoxetine treatments.

Fluoxetine treatment for three days					
Term and description	**Tot**	**Exp**	**Obs**	**P value**	**Enriched putative target genes**
GO:0001701 - In utero embryonic development	118	9	15	0,0384	Gja1, Tpm1, Plcg1, Smad2, Tgfbr1, Wnt9b, Rrn3, Ell, Ercc2, Prdm1, Tab1, Dusp32, Srsf1, Ube2b, Ihh
GO:0009791 - Post-embryonic development	42	3	9	0,0042	Cypd45, Smad2, Sepp1, Tgfbr1, Sema3c, Ccdc47, Morc3, Ercc2, Cfc1
GO:0006974 - Response to DNA damage stimulus	51	4	9	0,0153	Vav3, Atm, Bcl6, Ercc6, Nfatc2, Xpc, Obfc2b, Zmat3, Ube2b
GO:0032869 - Cellular response to insulin stimulus	40	3	8	0,0104	Ppat, D1a, Ahsg, Vldlr, Insig1, Ube2b, Pkcl, Rhoq
GO:0050853 - B cell receptor signaling pathway	13	1	5	0,0499	Pkcb, Syk, Vav3, Nfatc2, Hem1
GO:0009395 - Phospholipid catabolic process	8	1	4	0,0021	Pld1, Pld2, Plcg1, Pla2g4b
GO:0007610 - Behavior	13	1	4	0,0019	D1a, Glut, Usp46, Plcl1
GO:0045785 - Positive regulation of cell adhesion	14	1	4	0,0145	Pld2, Fgf1, Alox122, Vav3
GO:0006333 - Chromatin assembly or disassembly	16	1	4	0,0191	Egr2, Pbx1, Gdf10
GO:0043010 - Camera-type eye development	18	1	4	0,0307	Mitf, Neurod1, Mab21l2, Ihh
GO:0030890 – Positive regulation of B cell proliferation	18	1	4	0,0457	Vav3, Bcl6, Nfatc2, Nckap1l
GO:0060292 - Long term synaptic depression	5	0	3	0,0457	Hem1, Pirb1, Ikbkb
GO:0045089 - Positive regulation of innate immune response	6	0	3	0,0041	D1a, Neurod1, Emx2
GO:0030278 - Regulation of ossification	6	0	3	0,0078	Sav1, Lats2, Mobkl1a
GO:0001782 - B cell homeostasis	7	1	3	0,0078	Hem1, Pirb1, Ikbkb
Fluoxetine treatment for seven days					
Term and description	**Tot**	**Exp**	**Obs**	**P value**	**Enriched putative target genes**
GO:0007155 - Cell adhesion	191	55	70	0,0112	Gpnmb, Vcan, Nrsxn3, Nrxn2, Opcml, Nfasc, Pdcha4, Pdcha13, Cdh17, Fat3, Lamc2, Cgr11, Reln, Sdc4, Cd9, Scarb1, Sdc1, Gp5, Cntn2,

					Bcan, Cd44, Af6, Igfbp7, Adam9, Cdh9, Cd36, Cfdp1, Thbs2, Sell, Otog, Lyve1, Cpxm2, Cd47, Mpdz, Aoc3, Fermt1, Catna2, Catnal1, Maea, Ali1, Fer, Nid2, Igsf11, Vcl, Pcdh9, Catna1, Itga4, Lamc3, Col14a1, Pxn, Lmln, RGD1564327, Pcdha1, Pcdha2, Pcdha5, Pcdha6, Pcdha7, Pcdha9, Nrcam, Cdo, Adam17, Cspg3, Col5a3, Ctgf, Inppl1, Fat2, Cels3, Cyr61, Caspr, Cldn11
GO:0001701 - In utero embryonic development	118	34	48	0,0037	Icmt, Edn1, Ednra1, Eno1, Gad1, Cx43, Pdgfrb, Sp1, Tma2, Foxa2, Arnt2, Man2a1, Myr3, Nog, Notch1, Plcg1, Sox6, Zfp216, Tgfbr1, Cul3, Wnt9b, Tctn1, Rrn3, Ell, Rpgrip1l, Ercc2, Dnmt3l, Prdm1, Map3k7ip1, Slit2, Ncor2, Rai17, Gabpa, Maff, Egfl8, Med1, Dusp32, Byb1, Srf2, Aml1, Gata4, Tmed2, Sfrs1, Bmpr1a, Gna12, Ube2b, Cdh1, Ptprr
GO:0007268 - Synaptic transmission	58	17	25	0,0142	P2rx2, Stx1a, Nrxn3, Nrxn2, Cacnb2, Vglut1, Exoc4, Ap2b1, Cacna1c, Glut, Grm2, Grm4, Stx1b, Sstr1, Chat, GluA4, Cacna1g, GlR7, Lrp6, Sstr2, Csp, 5HT3, Acm2, Vdac1, Lis1
GO:0006814 - Sodium ion transport	63	18	25	0,0419	Vglut1, Ncx3, Ata1, Atp1a1, Atp1a2, Atp1a3, Atp1b2, Scnn1g, Slc9a2, Slc9a4, Scnn1a, Atp1b3, Vglut3, Tmprss8, Ata2, Scn11a, Slc22a5, Slc9a8, Scn6a, Scn2a1, Scn1a, Slc12a2, Slc24a2, Slc24a3, Slc5a7
GO:0009612 - Response to mechanical stimulus	47	14	23	0,0028	Txnip, Ets1, Igf1, Il6, Reln, Cxcl12, Mgp, Cav1, F31, Ptk2, Rcan1, Dcn, Cd36, Col1a1, Map1b, NFkB, Meis2, C-fos, Mapk9, Ctsb, Mmp2, Mmp9, Agpt2
GO:0030182 - Neuron differentiation	54	16	22	0,0405	Nsp, Map2k1, Gpc2, Atp2b2, Rb1, Casp3, Notch1, Ppia, Chat, Vgf, Pigt, Natc1, Itm2c, Otx2, Bhlhb5, En2, Runx1, Rxrg, Gas7, Ptprr, Efna1, Nnat
GO:0007409 - Axonogenesis	39	11	19	0,0068	Bcl2, Drd2, Snap25, Cntn2, Notch1, Ptpz, Igf1r, Slitrk6, Shootin1, Map1b, Ntng1, Prg12, Catna2, Slitrk1, Ndn, Ntng2, Slit2, Klf7, Dcamkl1
GO:0051591 - Response to cAMP	43	12	19	0,0229	Dusp1, Bcl2, Il6r, Sparc, Sdc1, Aldh3, Plat, Perc, Col1a1, Vgf, Pbp, NFkB, C-fos, Cdk2, Carm1, Foxm1, Duox2, Thbd, Tek
GO:0051726 - Regulation of cell cycle	37	11	18	0,0085	Uba3, Bcl2, Ptprc, Rb1, Gadd45, Mnt, Gadd45g, Ccng2, Skp2, Sgk, Bop1, Bap1, Ccne2, E2f1, Pten, Ccnd1, Nucks, Rb2, Cdk4
GO:0009887 - Organ morphogenesis	36	10	16	0,0333	Vegfc, Bcl2, Igf2, Insr, Titf1, Igf1r, Hhip, Pbx1, Otx2, NFkB, Traf6, Med1, Grem1, Hex, Megf5, Bmp7

GO:0000082 - G1/S transition of mitotic cell cycle	21	6	12	0,0062	Eif4e, Cdc25a, Bcl2, Calna1, Rb1, Skp2, Lats2, G2a, E2f1, Frap1, Ccnd1, Cdk4
GO:0046686 - Response to cadmium ion	21	6	11	0,0197	Prn, Sparc, Gss, Dmt1, Pcna, Hsd3b11, Mtf1, Cdk2, Mapk9, Haao, Cyb5
GO:0035094 - Response to nicotine	22	6	11	0,0293	Aldo1, Bcl2, Drd2, Edn1, Igf2, Chrena5, Casp3, Gnat3, Slc7a11, Mmp2, Mmp9
GO:0030183 - B cell differentiation	19	5	10	0,0249	Bcl2, Lca, Cebpg, Pou1f1, Tpd52, Rag2, Nhej1, Adam17, Hex, Rag1
GO:0033344 - Cholesterol efflux	11	3	9	0,0004	Abcg5, Abcg8, Scarb1, Cav1, Soat2, Npc2, Abca5, Apoc2, Stx12

Table 3. Expression analysis of putative target genes of miRNAs modulate after treatment with desipramine or fluoxetine for three or seven days

Treatment for three days				
	Desipramine		Fluoxetine	
Gene	mRNA FC	Protein %	mRNA FC	Protein %
Arid4	FC = -1,08 p = 0.39		FC = -1.03 p = 0.704	
Bcl6	-	-	FC = -1,42 P = 0.02*	-0.3% p = 0.972
Dnmt1	-	-	FC = 1,01 p = 0.935	
Galr1	-	-	FC = 2.97 **p = 0.018***	+0.5% p = 0.98
Smad2	FC = -1,09 p = 0.16	-30,26% **p = 0.0067***	FC = 1.01 p= 0.92	-19,55% **p = 0.048***
Tgfbr1	-	-	FC = -1.05 p = 0.51	-3.56% p = 0.81
Treatment for seven days				
	Desipramine		Fluoxetine	
Gene	mRNA FC	Protein %	mRNA FC	Protein %
Adar2	FC = -1.06 p = 0.46	+33.10% **p = 0.02***	FC = -1.22 p = 0.10	+9.19% p = 0.30
Bmp7	FC = 1,043 p = 0,88	-32.56% **p = 0.02***	FC = -1.348 p = 0.38	-21.77% p = 0.051
GalR1	-	-	FC=1.089 p = 0.78	-7.00% p = 0.41
Klf7	FC = -1.06 p = 0.34	+16.19% **p = 0.006***	FC = -1.08 p = 0.24	-3.94% p = 0.54
Kv4.2	FC =1.013 p = 0.97	-23.22% **p = 0.002***	FC = 1.049 p = 0.61	-7.38% p = 0.32
Smad1	FC = -1.247 p = 0.13	+20.00% **p = 0.03***	FC = -1.183 p = 0.19	+7.74% p = 0.14
TgfbR1	FC = -1.201 **p = 0.04***	-19.05% p = 0.29	FC = -1.132 p = 0.06	-5.21% p = 0.75
Zip1	FC=1.067 p = 0.56	-15.16% p = 0.14	FC = 1.109 p = 0.42	-6.69% p = 0.46

CHAPTER II

Early and time dependent modulation of hippocampal miRNome by agomelatine treatment

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ABSTRACT

MicroRNAs (miRNAs) have recently emerged as key regulators of complex temporal and spatial patterns of gene expression. MiRNAs are small (20–25 nucleotides) non-coding RNAs, that, can inhibit the translation of proteins and destabilize their target mRNAs, by binding to the target RNAs (mRNAs) in the 3´-UTR. MiRNAs may act as key regulators in a large number of different cellular processes, including early neurodevelopment, neuronal proliferation, apoptosis, metabolism, and cell differentiation. Recent studies suggest that miRNA may have a key role also in the pathophysiology and pharmacotherapy of neuropsychiatric disorders. Altered expression of miRNAs have been reported in both post mortem brain and peripheral tissues of patients with mood disorders, schizophrenia or other disorders of the brain. Recent lines of evidence also suggest a possible involvement of miRNAs in the action of psychotropic drugs, such mood stabilizers, antidepressants or antipsychotics.

Therefore, main aim of the present study was to investigate the possible involvement of miRNAs in the pharmacological action of agomelatine, by screening the complete miRNome with TaqMan array, in hippocampus of rats treated with agomelatine for different time lengths (3, 7 and 21 days). We found that agomelatine induces early and time-dependent effects on hippocampal miRNome expression profile, with the highest number of miRNAs modulated after 3-7 days of treatment (34 and 22, respectively). The bioinformatic analysis of miRNA target genes revealed early action of agomelatine in pathways involved in transcription, translation and epigenetic mechanisms (including circadian clocks), and later action on downstream mechanisms of neurotransmission and neuroplasticity.

INTRODUCTION

MIcroRNAs: a role in pathophysiology and pharmacotherapy of mood disorders

MicroRNAs (miRNAs) have recently emerged as key regulators of complex temporal and spatial patterns of gene/protein expression changes and, thereby, neuroplasticity (Corbin et al, 2009; McNeill and Van Vactor, 2012; Tardito et al., 2013). Converging evidence has suggested that the expression of the miRNome (the complex of all miRNAs expressed in a tissue at a given time) is highly regulated in a time- and region-specific manner both in the central nervous system (CNS) and in peripheral tissues. MiRNAs are small (20–25 nucleotides), non-coding RNAs partially complementary to messenger RNAs (mRNAs); by binding to the target transcript in the 3′-UTR, miRNAs can mainly inhibit the translation of proteins and destabilize their target mRNAs (Baek et al., 2008; Huntzinger and Izaurralde, 2011). Predicted to regulate about two thirds of the human genome, accumulating evidence indicate that miRNAs may act as key regulators in a large number of different cellular processes, including early neurodevelopment, neuronal proliferation, apoptosis, metabolism, and cell differentiation (Huntzinger and Izaurralde, 2011). A variety of miRNAs have been found in the CNS, and are believed to play critical roles in brain development and structural plasticity (McNeill and Van Vactor, 2012; O'Carrol and Schaefer, 2013). Recent evidence suggests that miRNAs regulation may prove to be key to our understanding of the pathophysiology and, ultimately, therapeutics of psychiatric disorders (Tardito et al., 2013; Kolshus et al., 2014). Recent evidence in post-mortem brain studies showed an overall decrease of miRNA expression in the prefrontal cortex (PFC) of schizophrenic (SCZ) subjects (Miller et al., 2012). Other authors described an increase in miRNA expression in temporal regions of SCZ patients, associated to a dysregulation of the biogenesis cofactor DGCR8, one of the candidate susceptibility loci for SCZ (Beveridge et al., 2008, 2010). More

recently, an overall decrease in miRNA expression was observed in PFC of depressed subjects committing suicide, with significant modifications in 21 miRNAs (Smalheiser et al., 2012).

Interestingly, recent findings suggest that some miRNAs and their targets are modulated by mood stabilizer agents (Zhou et al., 2009) or antidepressant drugs (Baudry et al., 2010; Bocchio-Chiavetto et al., 2013). In particular, it has been shown that selected miRNAs are regulated in hippocampus by prolonged lithium and valproate administration and that predicted effectors of common miRNA targets of lithium/valproate could participate to neurogenesis, neurite outgrowth, and nervous system development (Zhou et al., 2009). The first study assessing the possible involvement of miRNAs in the action of antidepressants was conducted by Baudry et al. (2010), who suggested a role for miR-16 in the mechanism of action of fluoxetine. Specifically, miR-16 appeared to create new serotonin sources in the brain through the switch of noradrenergic neurons toward a serotonergic phenotype. Recently, acute treatment with ketamine (an NMDA receptor antagonist shown to induce rapid and sustained antidepressant effect), electroconvulsive shock therapy and chronic fluoxetine treatment were described to reverse the changes in rat hippocampal miRNA expression induced by early life stress (O'Connor et al., 2013). The first study on peripheral miRNA expression in major depressed patients was conducted by Bocchio-Chiavetto et al. (2013), by evaluating the changes in global miRNA levels in whole blood after a 12-week effective treatment with the antidepressant drug escitalopram. A modulation was observed for 30 miRNAs; interestingly, target gene prediction and pathways analysis showed that these miRNAs might be implicated in several pathways associated with brain functions, such as neuroactive ligand–receptor interaction, axon guidance, long-term potentiation and depression, supporting the hypothesis of their involvement in the antidepressant mechanism.

Regulation of the biological clock by microRNAs

The biological implications of miRNAs are extended further by the recent discovery of their participation in the posttranscriptional control of circadian rhythms (Alvarez-Saavedra et al., 2011; Cheng et al., 2007). MiRNAs are expressed in the suprachiasmatic nuclei (SCN), the master circadian clock in mammals, in a rhythmic and inducible fashion, and modulate the intrinsic pacemaker activity and resetting capacity of the SCN (Cheng et al., 2007). In particular, it was shown that brain-specific miRNAs modulate the circadian clock located in the SCN by regulating clock-gene expression and the entraining effects of light. These effects are a result of the inducible translation control performed by miRNAs. However, little or no work of this kind has been done in areas other than the SCN, such as hippocampus and cortical areas.

Agomelatine and miRNAs

Agomelatine is a synthetic analogue of melatonin that is a potent agonist of melatonergic MT1 and MT2 receptors and antagonist of the serotonin 5-HT2C receptor subtype (Millan et al., 2003), with no affinity for a large number of other receptors studied. This unique pharmacological profile affords antidepressant-like activity via regulation of circadian rhythms. Importantly, in addition to SCN, both the melatonergic receptors and the 5-HT2C receptor are expressed in other brain areas involved in the pathophysiology of depression. Binding of agomelatine to MT1 receptors decreases receptor density and decreases firing of hypothalamic SCN cells, in a manner similar to melatonin. However, unlike melatonin, agomelatine interacts with 5-HT2C receptors, which are abundant in the SCN, frontal cortex, hippocampus, basal ganglia and other structures involved in mood, motor and cognitive deficits associated with depressive state. The antagonistic activity of agomelatine at the 5-HT2C receptor enhances frontocortical adrenergic and dopaminergic transmission (Popoli, 2009).

Based on this, aim of this work was to investigate whether miRNAs may be effectors of the antidepressant action of agomelatine. To this aim, the miRNome expression profile was assessed in hippocampus of rats treated with agomelatine; in order to verify a possible time-dependent effect of this drug, the animals have been treated for different time lengths: 3, 7 and 21 days.

EXPERIMENTAL PROCEDURES

Animals and drug treatments

Adult male Sprague-Dawley rats (170-250 g at the beginning of the study; Charles River, Calco, Italy) were kept at constant temperature (22°C) with a regular 12h light/dark cycle (light-on at 7 am). The rats were housed in groups of three (cages 45X28X20) with ad libitum access to food and water. Animals were habituated to housing conditions for 1 week before the beginning of experimental procedures. All animal procedures were conducted according to current regulations for animal experimentation in Italy (Decreto Legislativo 116/1992) and the European Union (European Communities Council Directive 2010/63/EU), were approved by the Italian Ministry of Health (Decreto Legislativo 295/2012-A) and the whole study adheres to the ARRIVE guidelines.

The rats, 9 for each experimental group, were treated with agomelatine (40 mg/kg i.p.) or vehicle (HEC 1%, 1ml/kg, i.p.) for 3, 7 or 21 days. Treatments were given at 5 pm (2 h before the start of dark cycle, 7 pm).

All the animals were sacrificed at the same age, the day after the last drug administration. Hippocampi (HPC) were quickly excised on ice as previously described (Tardito et al, 2009). Tissues were stored at -80° until subsequent use.

TaqMan array screen of agomelatine-induced microRNA expression

Total RNA including miRNA was extracted and purified from each hemi-hippocampus (randomly right or left) by using mirVana™ miRNA Isolation Kit following the manufacturer's protocol (Life Technologies BV Europe, Monza, Italy). RNA quantity and quality were assessed by using a NanoVue plus spectrophotometer (GE Healthcare, version 4282 V1.7.3).

150 ng of total RNA were then retrotranscribed using the TaqMan MicroRNA Reverse Transcription Kit and Megaplex™ RT Primers Sets A & B (Life Technologies) to synthesize single-stranded cDNA from total RNA samples. The cDNAs were then preamplified by using Megaplex PreAmp Primers Sets A & B (Life Technologies) in order to increase the quantity of miRNA cDNA for gene expression analysis. After preamplification, Quantitative Real Time PCR (qRT-PCR) was performed by using TaqMan Array Rodent MicroRNA A+B Cards Set v3.0 (Life Technologies) on Applied Biosystem Fast 7900HT, following the Applied Biosystems TaqMan Low Density Array experimental standardized protocol (50°C for 2 min, 94,5°C for 10 min, followed by 40 cycles of 97°C for 30 sec and 59,7°C for 1 min). TaqMan Array Rodent MicroRNA A+B Cards Set v3.0 (Sanger miRBase v15) is a two card set containing a total of 384 TaqMan® MicroRNA Assays per card as well as endogenous controls. An equal number of control and treated samples were processed and assayed on the same day, by an individual who was unaware of group identity. Raw Ct values were extracted from filtered SDS files by using the Applied Biosystem SDS 2.3 software; Ct values were normalized by the ΔΔCt method on endogenous controls U6B, U87, Y1 and snoRNA135, whose mean value did not vary across groups. A total of about 400 miRNAs were detected in all samples (mean Ct value <35).

Statistical analysis for miRNA expression

Statistical analysis was performed by using SAM software (Significance Analysis of Microarrays, version 4.0, Stanford University, http://www-stat.stanford.edu/~tibs/SAM/), which estimated statistical significance by subjecting the data to multiple random permutations. Indeed, SAM uses repeated permutations of the data to determine if the expression of any miRNA is significantly related to the response. The cutoff for significance is determined by a tuning parameter delta, chosen on the base of the false positive rate (FDR). In this study parameters were set for two-class unpaired analysis, Student's t-test statistic and 5000 permutations. FDR was set at <5%.

Bio-informatic analysis for the identification of miRNA putative target genes and functional annotation analysis

The putative target genes for each miRNA were determined by aggregating results from different prediction tools. We performed the analysis of the target site using the seed of the miRNA experimentally tested and searching them genome-wide against the dataset 3'-UTRs from the rat genome Rnor_5.0/ GCA_000001895.3, by employing different prediction tools such as: miRandav3.3a (John et al., 2005), TargetScanv5.1 (Lewis et al., 2005), RNA-hybrid v2.0 (Rehmsmeier et al., 2004), PicTar4way (Krek et al., 2005), DIANA-microT (Maragkakis et al., 2009) and PITAv6 (Kertesz et al., 2007). This approach was necessary since the TaqMan® Array Rodent MicroRNA included, besides Rattus Norvegicus, also included miRNA from human and mouse. To select predicted interactions, only 7–8 nucleotide matches were considered and only 1 G:U wobble base-pair and/or 1 mismatch was allowed. Results considered should be predicted by at least one algorithm plus PITA, with a negative binding energy.

The integration of these predictions has been performed by following a previously published approach of list re-ranking (Corrada et al., 2011) which overcomes the mere intersection or union of the results provided by the different algorithms mentioned above. This method has been further improved by changing the re-ranking algorithm, as implemented by the RankAggreg R package (Pihur et al., 2009). In detail, the best miRNA target list is defined as the simultaneously closest as possible to all the individual ordered lists provided by the different prediction algorithms, according to the Sperman footrule distance, that consists in the summation of the absolute differences between the ranks of all unique elements from both the original list and the optimized one. The lists produced by each algorithm ranged from 80 to 300 predictions and the integrated lists were computed by applying a deep optimization of the miRNA lists through the GA (generation=30000, popsize=1000) provided by the RankAggreg package, in order to achieve the best target::miRNA predictions. The algorithm always converged for the top 100 results, which were used for the following analysis.

The lists re-ranking performed with the GA allows to score the final target::miRNA list (the fitness of the list according to the final iteration of the GS), which provides an idea of the original consensus of the prediction obtained with the different algorithms.

The global analysis of the regulatory impact of miRNA requires methods to highlight the most significantly affected cellular processes. In this context, to gain information about the biological networks regulated by the identified miRNAs, the target gene lists were characterized relying on functional annotation against the three domains of the Gene Ontology (Biological Process, Cellular Component and Molecular Function), computed by using Biomart (Haider S et al, 2009). Moreover, annotations according to KEGG pathway terms (Kanehisa and Goto, 2000) was also conducted.

The associations were classified by assigning a statistical significance relying on an enrichment analysis approach. The probability from hypergeometric distribution was computed for each annotation term (meaning both nodes and leaves). We assigned an estimate (p-value) to the hypothesis that a pool of selected target genes actually belongs to a specific annotation term more than what could be found simply by chance, following the model proposed by Tavazoie et al. (1999). Fisher's exact test was used to statistically measure the over-representation of specific biochemical pathways, by comparing the distribution of the GO term in the population and in the sample. In detail, the total number of genes annotated with a specific GO term (tot) is used to calculate the expected value in the sample (exp), which is then statistically compared with the effective GO annotations observed in the sample (obs) to compute their enrichment and therefore their statistical significance in the sample.

Moreover, by employing semantic similarity we performed a clustering evaluation of the GO annotation terms. Cluster analysis was applied, relying on ontology similarity (Pesquita C et al., 2009) and performed through the GOSemSim package (Yu G et al., 2010) of R. The Cluster silhouette measures were considered in order to describe how appropriately single observations are grouped together. Filtering out clusters of annotation terms characterized by negative silhouette values (probably heterogeneous) helped in further enriching the top of the target lists, keeping only targets annotated with the most relevant terms. Finally, in order to provide a graphical visualization of GO terms identified, heat maps were computed for each branch of GO.

RESULTS

Effects of agomelatine treatments on hippocampal miRNome expression profile

In order to gain further insight in the mechanism of action of agomelatine and to understand whether miRNAs may mediate its action, rats were treated with agomelatine for 3, 7 or 21 days,. Hippocampal miRNome expression profiles were evaluated by using TaqMan array rodent MicroRNA A+B Cards, that allow the simultaneous quantization of 750 miRNAs. The expression analysis showed that a mean of about 450 miRNAs are expressed at good/detectable levels (CT<35) in each sample.

As shown in figure 1, the expression analysis of agomelatine group compared to the respective vehicle treated control group revealed a significant effect of the drug on miRNome expression profile at each time point assessed. Agomelatine showed a more pronounced effect after 3 days of treatment, with 34 miRNAs modulated: 6 up-regulated and 28 down-regulated (Figure 1a). Also after 7 days of treatment, high number of miRNAs were modulated by the antidepressant: a total of 22 miRNAs were modulated, with 3 up-regulated and 19 down-regulated (Figure 1b). Finally, a lower number of miRNAs were modulated after 21 days of treatment: 4 miRNAs up-regulated and 2 down-regulated (Figure 1c). Table 1 shows the fold changes and q values (SAM analysis, expressed in percentage value) of the miRNAs differentially expressed with respect to vehicle treated animals at all time points considering a FDR<5%.

Interestingly, 4 miRNAs (miR-463*, miR-632, let-7e* and mir-136*) were down-regulated by both 3 and 7 days of agomelatine treatment, with a greater effect observed with the longer treatment (Table 2). Another miRNA (miR-190b) was significantly modulated at both time points, but in opposite ways (Table 2). Moreover, miR-27a*, that was down-regulated after 7 days of agomelatine treatment, was up-regulated after 21 days (Table 2).

Computational analysis of miRNA targets and pathways

miRNA Target Prediction

A single miRNA can target hundreds of mRNA transcripts for either translation repression or degradation and computational prediction of miRNA targets is a critical initial step in identification of miRNA:mRNA target interactions for experimental validation. To this aim, we used a specifically designed computational approach, relying on a previously described method (Corrada et al., 2011). Potential target genes of the miRNAs significantly modulated by antidepressant treatments were identified by using different prediction tools: miRandav3.3a (John et al., 2005), TargetScanv5.1 (Lewis et al., 2005) RNA-hybrid v2.0 (Rehmsmeier et al., 2010), PicTar4way (Krek et al., 2005), DIANA-microT (Maragkakis et al., 2009) and PITAv6 (Kertesz et al., 2007). The predictions computed by each algorithm ranged from 80 to 300 results, which were integrated to provide an optimal target::miRNA list of predictions, as described in the Methods section, with convergence for the top 100 targets for each miRNA (Table S1).

Interestingly, some of the putative target genes, such as VEGF, SNARE proteins, Bcl2/6, ERK1/2, SNARE proteins, Bmal1 and Per2, key components of glutamate transmission (i.e. AMPA, NMDA and metabotropic glutamate receptors), interleukins, MAP1B, Ppp1r9b, Kynurenine 3-monooxygenase, TGFß pathway components (i.e. Tgfßr1, Tgfßr3, Tgfß2 and 3, Smad proteins), Wnt pathway components (i.e. Wnt9b, Wnt6, Wnt2b) among others, are already known to be involved in the mechanism of action of antidepressants, including agomelatine, and/or in the pathophysiology of mood and anxiety disorders (Krieglstein et al., 2011; Okamoto et al., 2010; Tardito et al., 2012). A specific bioinformatic analysis was also performed in order to identify putative targets commonly modulated by different miRNAs. The greater number of common

targets for at least three different miRNAs were found for miRNAs significantly modulated by 3 days of agomelatine treatment (n = 141; Table S2), also consistent with the higher number of miRNAs regulated (34) at this time point. Regarding 7 days of treatment with agomelatine, a mean of 4 different genes were found to be putative targets of at least three different miRNAs (Table S3). Instead, after 21 days of agomelatine treatment, no common targets were found for at least three different miRNAs; the highest number of miRNAs modulating the same gene was only 2 (data not shown), perhaps due to the low number of miRNAs regulated (6) with respect to the other time points.

Functional annotation and Enrichment Analysis

In order to identify the molecular and functional annotations and canonical biological pathways potentially influenced by target genes of miRNAs differentially expressed after treatment with agomelatine, we annotated the results using Biological Process, Cellular Component, and Molecular Function domains of Gene Ontology (GO), exploiting also the KEGG pathways database (Kanehisa and Goto, 2000) for a network analysis. By using these annotations, enrichment analysis was performed, in order to identify the most represented pathways and processes in which the identified miRNA target genes are involved. All the data regarding the three GO domains (Biological Process, Cellular Component and Molecular Function) as well as the KEGG pathways can be found in the Supplementary materials (Table S4). The ranking of the different biological processes in the tables was based on the higher number of genes observed for each term and on the smallest p-value. As highlighted by these lists, an enrichment in pathways associated to a wide range of processes is detectable for all three time points. In details, after 3 days of treatment (Table S4), 79 pathways were identified as significantly enriched in putative target genes in the GO Biological Process domain. Of these, the first 2 terms are associated with DNA regulation (Regulation of transcription, DNA-dependent, 131

putative target genes of miRNAs significantly modulated; p= 0.025; Transcription, DNA-dependent, 48 putative target genes, p= 0.016), thus suggesting the possible involvement of epigenetic mechanisms; the third pathway identified is In utero embryonic development (42 putative target genes, p= 0.018). We then performed a further analysis in order to highlight the presence of significant clusters of the GO annotation term. As shown in the heat map (Figure 2a), 4 significant clusters were identified, the first associated to DNA-RNA regulation (Transcription, DNA-dependent; Regulation of transcription, DNA-dependent and Negative regulation of gene-specific transcription from RNA polymerase II promoter), the second associated to the regulation of cell adhesion (Negative regulation of cell adhesion, Negative regulation of cell cycle and Regulation of microtubule-based process), the third associated to the development and morphogenesis of different organs, including brain (Tube morphogenesis, Lung development, Embryonic heart tube development, Metanephros development, Cerebellum development, Thyroid gland development, Pancreas development, Salivary gland morphogenesis and Odontogenesis of dentine-containing tooth), and the fourth associated to the morphogenesis of axons and dendrites (Cell morphogenesis, Axon target recognition and Dendrite morphogenesis). The heat map and cluster analysis relative to the other GO domains (Cellular Component and Molecular Function) for the 3 days treatment are reported in the Supplementary Section (Figure S1 a and b).

After 7 days of treatment with agomelatine, we found 117 pathways in the GO Biological Process domain significantly enriched in putative target genes. As shown in Table S4, the first three pathways in this domain are Transport (35 putative target genes, p= 0.026), In utero embryonic development (31 putative target genes, p= 0.018) and Positive regulation of cell migration (17 putative target genes, p= 0.022). Several pathways are correlated to

neurotransmission, as shown also by the cluster analysis. Indeed, this analysis revealed the presence of 5 main clusters (Figure 2b), and the one with the highest number of pathways is strictly related to the regulation of neurotransmission (Long-term synaptic potentiation, Negative regulation of synaptic transmission, GABAergic, Long term synaptic depression, Regulation of neuronal synaptic plasticity, Regulation of synaptic transmission, GABAergic, Regulation of synaptic transmission, glutamatergic). Two of the other clusters are correlated to the development of anatomical structures and organs (the first one includes: Anatomical structure development, Anatomical structure morphogenesis and Anatomical structure formation involved in morphogenesis; the second one includes: Urogenital system development, Metanephros development and Kidney development), while the remaining two are correlated to phosphorylation of proteins (Pathway-restricted SMAD protein phosphorylation, Protein modification process and Peptidyl- tyrosine phosphorylation) and to regulation of cell adhesion (Negatine regulation of cell adhesion, Regulation of cell-matrix adhesion and Negative regulation of cell matrix adhesion)(Figure 2b). The heat maps representing the most significant clusters for the GO domains of Cellular Component and Molecular Function are reported in Figure S2 a and b.

Regarding the longest time of treatment, 21 days, with a total of 48 pathways identified in the GO Biological Process domain (Table S4), the first three pathways are Transmembrane transport (28 putative target genes, p= 0.007), Positive regulation of transcription from RNA polymerase II promoter (23 putative target genes, p= 0.028) and Brain development (13 putative target genes, p= 0.013). Also at this time point, several pathways are correlated to neuronal functions, i.e. Calcium ion transport, Regulation of axonogenesis, Regulation of synaptic vesicle priming, L-glutamate transport and import. The cluster analysis has revealed

the presence of 3 main clusters, two of them correlated to the transport of different kind of molecules (the first one includes: L- amino acid transport, L- glutamate transport and L- glutamate import; the second one includes. Axon cargo transport, Mitochondrial transport, Endosome transport, Transmembrane transport, Vesicle-mediated transport and Transcytosis) and the other one correlated to brain development (Cerebellar Purkinje cell layer development, Brain development and Hindbrain development) (Figure 2c). Figure S3 (a and b) shows heat maps of significant clusters identified in the GO Cellular Components and Molecular Function terms.

Analysis for miRNA similarly modulated by agomelatine after 3 and 7 days of treatment

The presence of some miRNAs modulated by agomelatine at different time lengths (Table 2) could suggest a possible time-dependent modulation of related targets. In order to identify these possible targets, a similar bioinformatic analysis was performed on these specific miRNAs. Regarding the 5 miRNAs modulated by both 3 and 7 days of agomelatine treatment, we found a significant enrichment of miRNA putative target genes in 106 pathways belonging to Biological Process, 10 pathways to Cellular Component, 43 to Molecular Function GO domains and 5 KEGG enriched pathways (Table S7). In the Biological Process GO domain, the first three terms identified are Regulation of transcription, DNA-dependent (21 putative target genes, p= 0.019), In utero embryonic development (10 putative target genes, p= 0.003) and Nervous system development (8 putative target genes, p= 0.041). Interestingly, some pathways are correlated to DNA/RNA regulation mechanisms (i.e. Negative regulation of translation, Posttranscriptional regulation of gene expression, mRNA methylation) while other pathways are correlated to central nervous system functions (i.e. Positive regulation of calcium ion transport and Central nervous system interneuron axonogenesis). As shown in Figure 2d, the cluster analysis for the Biological Process GO domain highlighted the presence of 2 main

clusters, both correlated to the development and differentiation of organs (the first one includes: Cardioblast differentiation, Vasculature development, Heart development and Embryonic heart tube development; the second one includes: Thyroid gland development, Lung development, Inner ear development, Liver development, Nervous system development and Hindbrain development). A significant cluster was found also in the Molecular Function GO term (see Figure S4), but not in the Cellular Component term. The bioinformatic analysis performed on the single miRNA modulated after 7 and 21 days of treatment showed the presence of pathway with poor enrichment, perhaps due to the small number of target (only a miRNA is involved: data not shown).

miRNAs and putative targets related to circadian rhythms

By using a candidate approach based on bioinformatics analyses and data in the literature (Yamaguchi et al., 2000; Li et al., 2013; Figueredo et al., 2013), we found that some of the miRNAs modulated by agomelatine treatments could regulate target genes involved in the control of circadian rhythms (Table 3). Some interesting consideration could be done: first, the great number of miRNAs and target genes was found after treatment for 3 and 7 days with agomelatine, suggesting a possible early modulation of circadian rhythms by agomelatine (Table 3). Second, some of the miRNAs are significantly modulated after different length of treatment with agomelatine: i.e, let7e was decreased after both 3 and 7 days of treatment; miR-632 was decreased by 3 days of treatment and virtually abolished after 7 days of agomelatine; miR190 was reduced by 3 days of treatment and increased after 7 days and finally, miR-27 was reduced after 7 days and increased after 21 days.

DISCUSSION

Early and time-dependent effects of agomelatine on hippocampal miRNome expression profile

Our results showed for the first time that agomelatine induces early and time-dependent effects on hippocampal miRNome expression profile. Indeed, a differential modulation of miRNA expression was observed after the three lengths of treatment. In particular, a significant modulation of 34 miRNAs was found after treatment with agomelatine for three days. Interestingly, as shown in Figure 1a, most of the miRNAs were downregulated (28 vs. 6) with fold changes ranging from -1.42 to -6.23 and from 1.48 to 2.60 for the 6 miRNAs that were upregulated (Table 1). As shown in Figure 1b, agomelatine significantly modulated the expression of 22 miRNAs after seven days of treatment, reducing again the expression of most of them (20). Interestingly, five miRNAs were found to be modulated after both time of treatment: miR-632, Let-7e*, miR-463, miR-136* and miR-190b. Four of them were reduced after both 3 and 7 days of treatment, whereas miR190b was significantly reduced after 3 days and markedly increased after 7 days of treatment (Table 2). miR-632, that was slightly but significantly reduced by 3 days of agomelatine treatment, was almost abolished by 7 days of treatment. Finally, only 6 miRNAs were found to be differentially expressed with respect to vehicle treated animals after 21 days of agomelatine treatment (Figure 1c). In this case, agomelatine reduced the levels of two miRNAs and increased the expression of 4 miRNAs; among these, miR-27a was found to be reduced after 7 days of treatment (Table 2).

These results suggest that miRNAs could have a role in the action of agomelatine and suggest that miRNAs may represent early mediators of agomelatine effects.

Bioinformatics analysis: putative target genes and pathways involved

The bioinformatic analyses performed in order to identify putative target genes of miRNAs significantly modulated by agomelatine treatments produced lists of hundreds of genes (about 100 for each miRNA found to be modulated by agomelatine treatment). Among the putative miRNA target genes, several are already known to be involved in the mechanism of action of antidepressants: as an example, VEGF, different proteins of the SNARE complex; Bcl6; Erk1/2; p75; NMDA and AMPA glutamatergic receptors; eEF2, among others. Moreover, others have been related in particular to agomelatine action: SNARE proteins, Bmal1, Per2, different components of glutamatergic transmission, Interleukins, MAP2, Ppp1r9b, and kynurenine 3-monooxygenase (Musazzi et al., 2010; Racagni et al., 2011; Ladurelle et al., 2012; Molteni et al., 2013).

The annotation analyses performed with the GO subcategories Biological processes, Molecular functions, Cellular components and KEGG pathways, highlighted several significant pathways enriched in target genes of miRNAs modulated by agomelatine treatments. Although some of them were commonly found in the different treatments, others seem to be mainly related to one or two time of treatment. In particular, a higher presence of pathways related to mechanism of RNA or DNA regulation and transcription was found considering miRNAs modulated by 3 days of treatment with agomelatine with respect to the 7 or 21 days of treatment. Indeed, considering all the GO subcategories, among the significant cluster identified there were pathways such as: transcription- DNA-dependent, regulation of transcription - DNA-dependent, negative regulation of gene-specific transcription from RNA polymerase II promoter (Biological processes, Table S4); transcription factor TFTC complex, histone deacetylase complex, transcriptional repressor complex, condensed chromosome; nucleosome, replication fork (Cellular components, Table S4) and single-stranded DNA binding,

DNA binding, double-stranded DNA binding, histone deacetylase binding, transcription factor binding and SMAD binding (Molecular functions, Table S4). These results suggest that a short-term treatment with agomelatine, through the modulation of miRNA expression, could lead to rapid changes in the regulation of RNA/DNA dependent transcription of target genes involved in its mechanism of action. Moreover, several of these pathways are enriched in effectors of epigenetic mechanisms (such as histone proteins, histone deacetylase, DNA – methyltransferase, histone –methyltransferase, lysine-specific demethylase, among others), thus further supporting the involvement of epigenetic mechanisms in the action of antidepressant drugs (Menke et al., 2012; Sun et al., 2013; Tardito et al., 2013).

Another interesting result concerns the enrichment of several components of the transforming growth factor beta (TGFß) pathway among the putative target genes of miRNAs significantly modulated by 3 and 7 days of agomelatine treatment, but not after 21 days of treatment, again suggesting the selective time-dependent modulation of specific signaling pathways by agomelatine (Table S4). It is becoming increasingly clear that members of the TGFß family (i.e. TGFß, activin, bone morphogenetic proteins) have roles in the CNS that extend beyond their well-established roles as neurotrophic and neuroprotective factors (Krieglstein et al., 2011). Although the impact of bone morphogenetic proteins (BMP) signaling on central synaptic functions is only beginning to be deciphered, a growing body of evidence implicates TGFß/activin signaling pathways in the homeostatic regulation of excitatory and inhibitory synapses in the intact CNS, with particular emphasis on the mechanisms underlying short-term and long-term plasticity. Studies using transgenic mice demonstrate that malfunctions in TGFß/activin signaling cascades might disturb various features of synaptic transmission. At the behavioral level, loss of TGFß/activin signaling gives rise to a broad spectrum of behavioral abnormalities, including cognitive impairments, affective disorders and deficits in sensorimotor

gating. Thus, TGFß and activin signaling are not only involved in the protection of neurons after acute or prolonged brain damage, but might also be intimately involved in the daily operations of synaptic networks that are associated with cognition and emotional stability (Krieglstein et al., 2011; Ganea et al., 2012).

A picture somewhat different emerged from the bioinformatic analyses conducted on the miRNAs modulated by more prolonged treatments with agomelatine. The majority of pathways enriched in miRNA putative target genes after 7 or 21 days of agomelatine are indeed mainly related to neurotransmission and neuroplasticity (Table S4). Among the significative clusters identified in the Biological processes subcategory of the GO annotation analysis for targets of miRNAs modulated by 7 days of treatment we found pathways such as LTP and LTD, synaptic plasticity, glutamatergic transmission and GABAergic transmission (Table S4). This picture was even more evident considering the data obtained for the 21 days treatment, with an enrichment of miRNA putative targets in pathways such as calcium transport, synaptic vesicle priming, synaptosomes, neurotrophin signaling, synapse, axon cargo transport, GABAergic transmission, WNT signaling among others (Table S4).

Overall, these data suggest that, by modulating different networks of miRNAs, agomelatine treatments elicit an early action on mechanisms mainly involved in DNA/RNA modulation (i.e., transcription, translation, epigenetic mechanisms) and to a lower extent in neuroplasticity, whereas after longer time of treatment on pathways primarily involved in downstream mechanisms of neurotransmission and neuroplasticity. It is noteworthy that, based on the bioinformatic results, several other interesting pathways seem to be potentially involved in the effects of agomelatine thus suggesting the need for further investigations.

miRNAs and putative targets related to circadian rhythms

Several studies recently revealed that miRNAs, consistent with their canonical function as translational modulators of target genes and their widespread roles in cell physiology, have a functional impact on the circadian clock of mammals, either because of direct targeting of the core clock machinery or through more indirect means that ultimately feed into the circadian clock (Figueredo et al., 2013; Chen et al., 2013). By using a candidate approach based on bioinformatics analyses and data in the literature (Yamaguchi et al., 2000; Li et al., 2013; Figueredo et al., 2013), we found that some of the miRNAs modulated by agomelatine could regulate genes involved in the control of circadian rhythms (Table 3). Interestingly, most of them were found after treatment for 3 and 7 days with agomelatine, suggesting a possible early modulation of circadian rhythms by agomelatine (Table 3). Also, it is noteworthy a time-dependent regulation of some of the miRNAs: let-7e* was decreased after both 3 and 7 days of treatment; miR-632 was decreased by 3 days of treatment and "abolished" after 7 days of agomelatine; miR190 was reduced by 3 days of treatment and increased after 7 days and finally, miR-27 was reduced after 7 days and increased after 21 days, thus further supporting the hypothesis of a participation of miRNAs in the chronobiotic properties of agomelatine (Popoli, 2009; Racagni et al., 2011)

Role of the funding sources

Contributors

Conflict of interest

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Legends of Figures and Tables

Figure 1: MiRNAs significantly modulated by agomelatine treatment. A): Hippocampal miRNAs significantly modulated by 3 days of treatment with agomelatine. B) Hippocampal miRNAs significantly modulated by 7 days of treatment with agomelatine. C) Hippocampal miRNAs significantly modulated by 21 days of treatment with agomelatine.

Values on the y-axis are fold changes with respect to vehicle treated animals. SAM analysis (p<0.05, FRD<5%).

Figure 2: Hierarchical clustering of enriched annotations in the GO Biological process category for predicted targets of miRNAs modulated by agomelatine treatment.

The heat maps highlight significant clusters of the GO-Biological process terms enriched in putative target genes of miRNAs modulated by: (a) 3 day-treatment; (b) 7 day-treatment; (c) 21 day-treatment; (d) miRNAs modulated by both 3 and 7 day treatment. The color ramp of the heatmap is defined according to the values of similarity score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1). Green squares highlight significant clusters (described in the text).

Table 1: miRNAs significantly modulated by agomelatine treatment.

Fold changes and q-values (SAM analysis, FDR<5%, p<0.05) of miRNAs modulated by agomelatine treatment for 3, 7 and 21 days.

Q Value= lowest False Discovery Rate at which that gene is called significant.

Table 2: MiRNAs found to be significantly modulated at different time length of agomelatine treatment.

Fold changes of miRNAs modulated by agomelatine at different time lengths.

Table 3: miRNAs and putative target genes related to control of circadian rhythms. - = decreased by agomelatine treatments; + = increased by agomelatine

Table S1: Putative target genes of miRNAs modulated by agomelatine treatment.

Table reports the first 100 best targets predicted for each miRNA significantly modulated by 3, 7 and 21 days of agomelatine treatment.

In blu the score for each target::miRNA list (the fitness of the list according to the final iteration of the GS), that provides an idea of the original consensus of the prediction obtained with the different algorithms.

When present, at the end of each list are highlighted in red target genes experimentally validated elsewhere.

Table S2. Putative target genes modulated by more than 3 miRNAs.

In the table are listed putative target genes of more than 3 miRNAs for 3 days of agomelatine treatment.

Table S3. Putative target genes modulated by more than 3 miRNAs.

In the table are listed putative target genes of more than 3 miRNAs for 3 days of agomelatine treatment.

Table S4: Significantly overexpressed terms and enriched genes in the GO –categories and KEGG pathways for miRNAs modulated by agomelatine treatments

The file reports the overexpressed terms in the GO biological process, Molecular Function, Cellular Components and KEGG pathways, together with the respective enriched genes for miRNAs modulated by agomelatine treatment: a) 3 days (AGO 03); b) 7 days of (AGO 07); c) 21 days (AGO 21); d) miRNAs modulated by both 3 and 7 days (AGO 03-07).

Enriched terms in are ranked on the number of observed genes and p value. Tot = the total number of genes annotated with a specific GO term; Exp = the expected value of genes in the sample (exp); Obs = effective GO annotations observed in the sample. Statistics: Fisher's exact test.

Enriched genes are listed according to the alphabetical order of the term to which they belong.

Figure S1: Hierarchical clustering of enriched annotations in the GO Cellular Component and Molecular Function category for predicted targets of miRNAs modulated by 3 days of agomelatine treatment.

The heat maps highlight significant clusters of the Cellular Component and Molecular Functions GO terms enriched in putative target genes of miRNAs modulated by 3 days of agomelatine treatment: (a) Cellular component; (b) Molecular Function. The color ramp of the heatmap is defined according to the values of similarity score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1). Green squares highlight significant clusters.

Figure S2: Hierarchical clustering of enriched annotations in the GO Cellular Component and Molecular Function category for predicted targets of miRNAs modulated by 7 days of agomelatine treatment.

The heat maps highlight significant clusters of the Cellular Component and Molecular Functions GO terms enriched in putative target genes of miRNAs modulated by 7 days of agomelatine treatment: (a) Cellular component; (b) Molecular Function. The color ramp of the heatmap is defined according to the values of similarity score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1). Green squares highlight significant clusters.

Figure S3: Hierarchical clustering of enriched annotations in the GO Cellular Component and Molecular Function category for predicted targets of miRNAs modulated by 21 days of agomelatine treatment.

The heat maps highlight significant clusters of the Cellular Component and Molecular Functions GO terms enriched in putative target genes of miRNAs modulated by 21 days of agomelatine treatment: (a) Cellular component; (b) Molecular Function. The color ramp of the heatmap is defined according to the values of similarity score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1). Green squares highlight significant clusters.

Figure S4: Hierarchical clustering of enriched annotations in the GO Molecular Function category for predicted targets of miRNAs modulated by both 3 and 7 days of agomelatine treatment.

The heat maps highlight significant clusters of the Molecular Functions GO terms enriched in putative target genes of miRNAs modulated by both 3 and 7 days of agomelatine treatment. No significant clusters were found for the Cellular Component terms. The color ramp of the heatmap is defined according to the values of similarity score (blu=minimum similarity, red=maximum similarity). The ceiling threshold of the score is defined by coupling the same term (the red diagonal, value=1). Green squares highlight significant clusters.
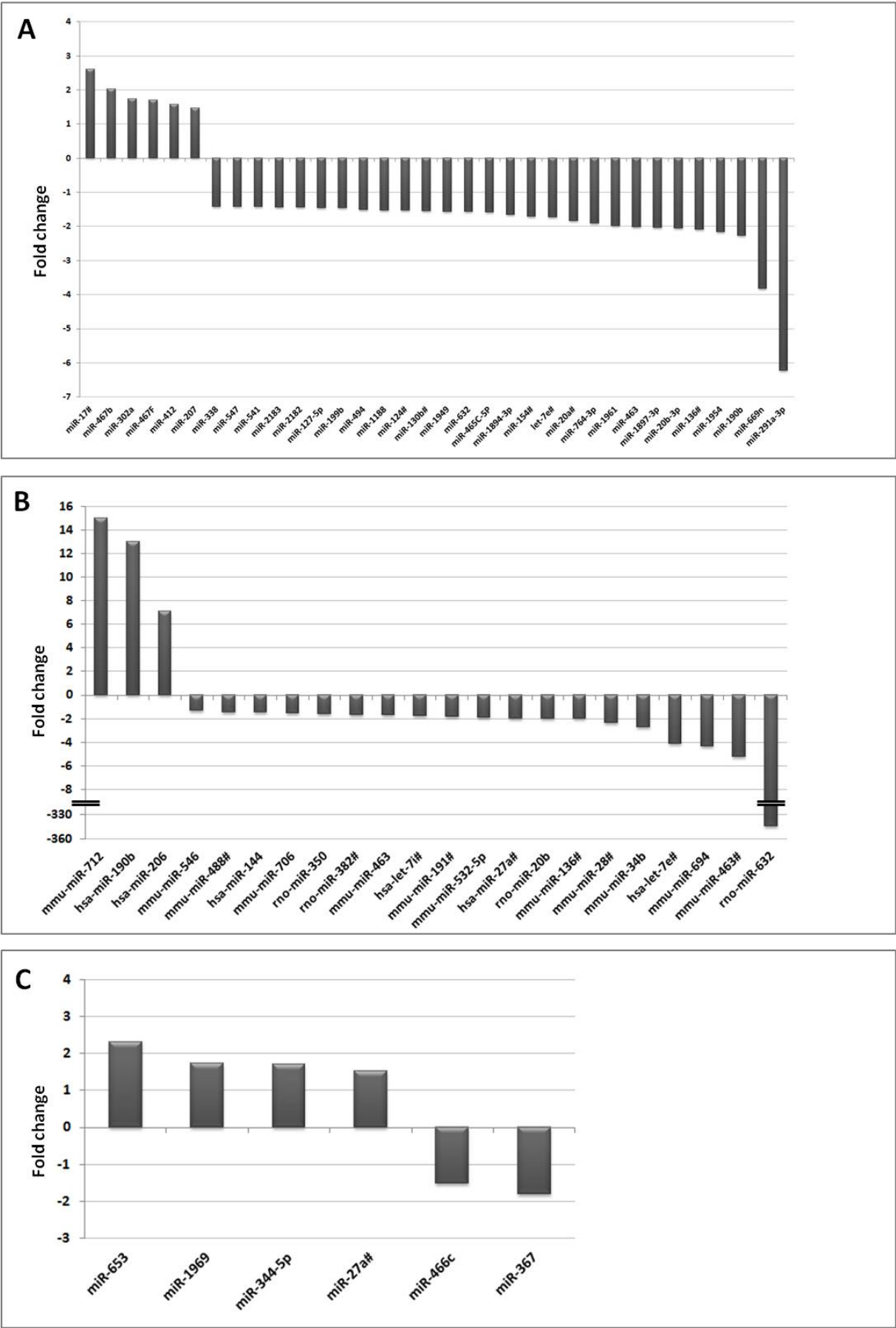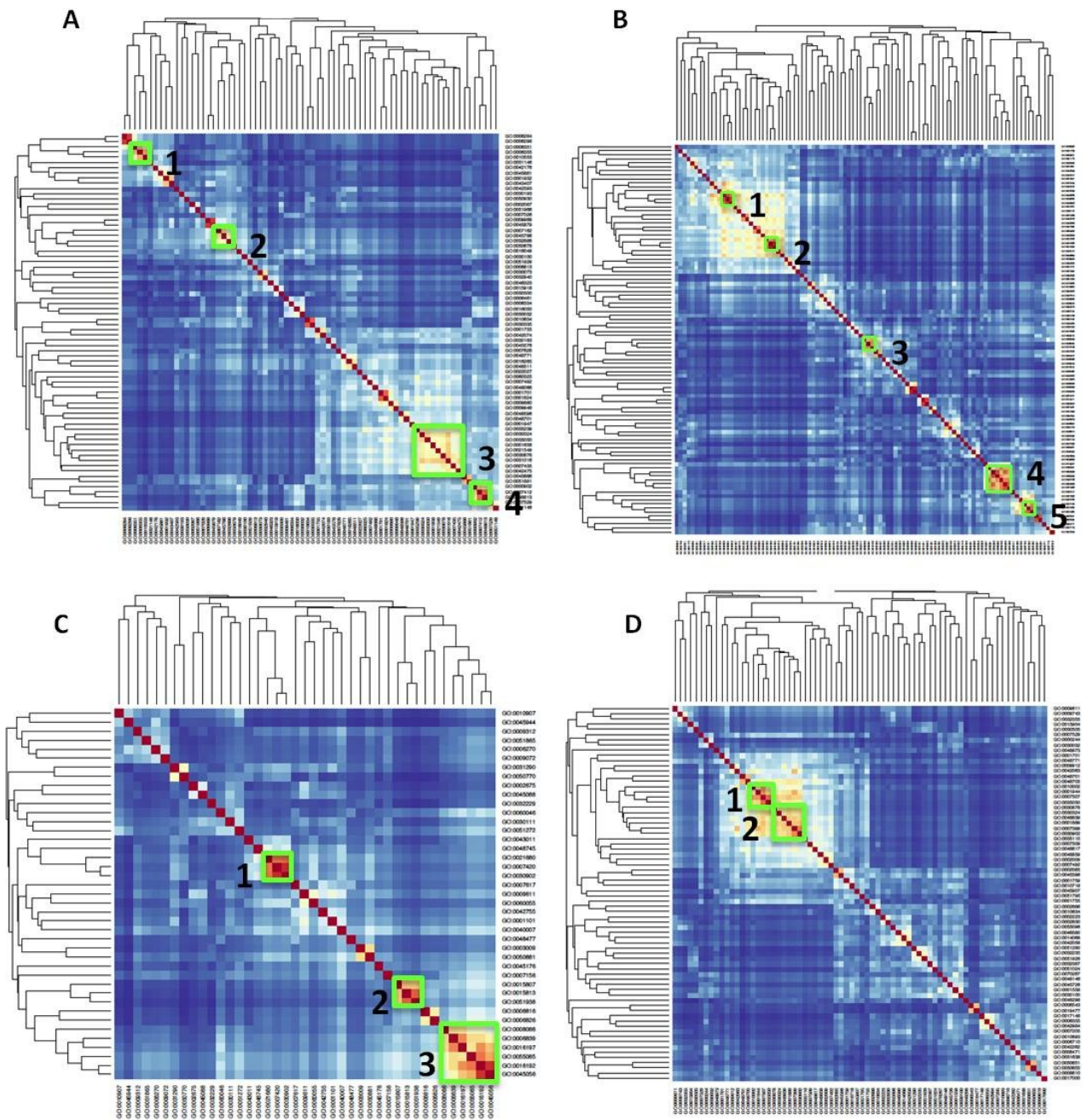
Figure 1

Figure 2

Table 1. MIRNAs significantly modulated by agomelatine treatment.

3 days of agomelatine treatment			7 days of agomelatine treatment			21 days of agomelatine treatment		
miRNA	FC	Q-Value (%)	miRNA	FC	Q-Value (%)	miRNA	FC	Q-Value (%)
miR-17*	2,60	0.00	miR-712	14,99	0.00	miR-653	2,30	0.00
miR-467b	2,02	0.00	miR-190b	12,98	0.00	miR-1969	1,73	0.00
miR-302a	1,74	0.00	miR-206	7,11	0.00	miR-344-5p	1,72	0.00
miR-467F	1,70	0.00	miR-546	-1,29	4.86	miR-27*	1,53	0.00
miR-412	1,58	0.00	miR-488*	-1,46	0.00	miR-466c	-1,53	0.00
miR-207	1,48	4.61	miR-144	-1,47	0.00	miR-367	-1,81	0.00
miR-338	-1,42	0.00	miR-706	-1,58	4.86			
miR-547	-1,43	4.61	miR-350	-1,59	0.00			
miR-541	-1,43	4.61	miR-382*	-1,70	0.00			
miR-2183	-1,43	0.00	miR-463	-1,72	0.00			
miR-2182	-1,44	2.90	let-7i*	-1,78	0.00			
miR-127-5p	-1,46	2.90	miR-191*	-1,86	0.00			
miR-199b	-1,46	2.90	miR-532-5p	-1,94	0.00			
miR-494	-1,52	2.90	miR-27*	-1,95	0.00			
miR-1188	-1,53	2.90	miR-20b	-2,00	0.00			
miR-124*	-1,54	4.61	miR-136*	-2,01	0.00			
miR-130b*	-1,55	4.61	miR-28*	-2,39	0.00			
miR-1949	-1,56	0.00	miR-34b	-2,72	4.86			
miR-632	-1,57	0.00	let-7e*	-4,11	0.00			
miR-465C-5P	-1,58	0.00	miR-694	-4,33	0.00			
miR-1894-3p	-1,66	0.00	miR-463*	-5,21	4.86			
miR-154*	-1,71	0.00	miR-632	-344,03	0.00			
let-7e*	-1,73	0.00						
miR-20*	-1,83	0.00						
miR-764-3p	-1,91	0.00						
miR-1961	-1,98	0.00						
miR-463	-2,02	0.00						
miR-1897-3p	-2,04	0.00						
miR-20b-3p	-2,05	0.00						
miR-136*	-2,09	0.00						
miR-1954	-2,16	0.00						
miR-190b	-2,28	4.61						
miR-669n	-3,83	0.00						
miR-291a-3p	-6,23	0.00						

Table 2: MiRNAs found to be significantly modulated at different time length of agomelatine treatment.

miRNA	3 days FC	7 days FC	21 days FC
miR-632	-1,57	-344,03	
Let-7e*	-1,73	-4,11	
miR-463	-2,02	-5,21	
miR-136*	-2,09	-2,01	
miR-190b	-2,28	12,98	
miR-27a*		-1,95	1,53

Table 3: miRNAs and putative target genes related to control of circadian rhythms.

miRNA 3 days	Putative targets	miRNA 7 days	Putative targets	miRNA 21 days	Putative targets
Let-7e -	mCry2; mRorc	Let-7e -	mCry2; mRorc	miR-27 +	mAdcy6, Npas2
miR-127 -		mir-27 -	mAdcy6, Npas2	Mir-367 -	Per2
miR-190 -		miR-190 +			
miR-302a +		miR-191 -			
miR-494 -	Bmal1	miR-632 --			
miR-632 -	Dbp		Dbp		

GENERAL DISCUSSION AND CONCLUSIONS

Mood Disorders and in particular MD, have been estimated to be the fourth major cause of disability worldwide and may become second only to cardiovascular diseases in the next two decades. Less that 50% of all patients treated with the currently available antidepressants (AD) show full remission and the therapeutic effects of AD in the treatment of MD are seen only after chronic administration. However, despite the clear need for better therapies, recent efforts to develop novel antidepressants have been relatively unsuccessful. A main reason for this is the still incomplete knowledge of pathogenetic mechanisms of MD and understanding of AD mechanisms. The present and updated version of the hypothesis on MD and AD action, namely the hypothesis of neuroplasticity, integrates postreceptor intracellular signaling cascades with the mechanisms of gene expression, posttranscriptional regulation and several other processes, including synaptic and neurotrophic mechanisms and neurogenesis.

In the past few years growing evidence has supported a key role for miRNAs in central nervous system (CNS) development and homeostasis. It has been reported that almost 50% of all identified miRNAs are expressed in mammalian brain, where they appear to be differentially distributed not only in specific areas but also within neurons and distinct neuronal compartments. This highly specific compartmentalization has been shown to be a key factor in the control of local protein expression, synapse development and function. A role for miRNAs in neurogenesis, neuronal differentiation and survival, as well as in neuroplasticity, is now well established (Mouillet-Richard 2012; Tardito et al., 2013;).

In our work we used a genome wide approach to assess whether miRNAs could have a role in the regulation of neuroplasticity in the response to therapeutic drugs for MD. In these studies we have indeed evaluated the effects on hippocampal rat miRNome of antidepressants with different mechanism of action: desipramine, fluoxetine and agomelatine. In order to better evaluate the possible time-dependent effects of these drugs, desipramine and fluoxetine have

been administered for 3 and 7 days, while agomelatine for 3, 7 and 21 days. Our results have shown for the first time a different, specific and time-dependent effect of antidepressants on miRNAs modulation. In particular, all drugs showed an early effect after 3 days of treatment, with major effects given by agomelatine (34 miRNAs modulated, mostly downregulated). Instead, at this time point, desipramine and fluoxetine modulated 8 different miRNAs in opposite ways (the first AD upregulating and the second downregulating them). After seven days of treatment, the greater effect was shown by fluoxetine, that modulated 35 miRNAs (mostly upregulated), while desipramine upregulated 13 miRNAs, and agomelatine modulated 22 miRNAs (mostly downregulating them). Finally, regarding the 21 days of treatment with agomelatine, only 6 miRNAs were found to be modulated. Interestingly, 8 miRNAs were similarly increased by desipramine and fluoxetine after seven days of treatment, thus suggesting the presence of common targets. Moreover, while no one of the miRNAs modulated by desipramine and fluoxetine after 3 days of treatment was significantly modified after 7 days of treatment, agomelatine modulated some miRNAs at different time lengths (5 miRNAs after both 3 and 7 days and 1 miRNA after both 7 and 21 days), thus suggesting a possible time-dependent modulation of related targets.

The bioinformatic analysis was performed using a specifically designed computational approach, relying on a previously described method (Corrada et al., 2011). The analyses produced lists of hundreds of genes. For all the three drugs, among the putative miRNA target genes, several are known for their involvement in the mechanism of action of antidepressants and/or in the pathophysiology of mood and anxiety disorders. Among them, some have also been previously related to agomelatine action, like the genes involved in circadian rhythm modulation (i.e. Bmal1, Per2). In order to identify the molecular and functional annotations and canonical biological pathways potentially influenced by target genes of differentially expressed

miRNAs after treatment with desipramine, fluoxetine or agomelatine we annotated the results using Biological Process, Cellular Component, and Molecular Function domains of Gene Ontology (GO), exploiting also the KEGG pathways database (Kanehisa and Goto, 2000), for a network analysis. Our results showed an enrichment of pathways related to a great variety of processes including metabolism of glucose and lipids, development of organs, intracellular and extracellular transport, etc. It is noteworthy that a great number of pathways were related to neuronal plasticity (i.e., regulation of synaptic transmission, LTP, LTD, synaptic vesicle docking, MAPK cascade, among the others). These pathways emerged at both times of treatment with desipramine and fluoxetine, and also with agomelatine, especially after the longer time lengths (7 and 21 days). A high number of pathways related to mechanism of RNA or DNA regulation was also found (i.e. regulation of transcription - DNA-dependent, histone deacetylase complex, transcriptional repressor complex among the others), in particular for 3 days of desipramine and agomelatine treatment, thus suggesting the presence of epigenetic mechanisms in the early action of antidepressant drugs (Sun et al., 2013; Tardito et al., 2013). Another interesting result concerns the enrichment of several components of the transforming growth factor beta (TGFß) pathway among the putative target genes of miRNAs significantly modulated by 3 and 7 days of desipramine, fluoxetine and agomelatine treatment, but not after 21 days of agomelatine treatment. Studies have reported that the members of the TGFß family (i.e. TGFß, activin, bone morphogenetic proteins) are involved in cognitive, behavioural and affective disorders, and have roles in the central nervous system in the homeostatic regulation of excitatory and inhibitory synapses and as neurotrophic and neuroprotective factors, (Krieglstein et al., 2011).

Regarding desipramine and fluoxetine treatments, some interesting putative target genes have been selected for validation through mRNA and protein expression studies. Some of them,

namely GalR1, Smad2, Tgfbr1, Bmp7 and Kv4.2 have showed expression changes coordinated with their regulating miRNAs. Other genes, Smad1, Adar2 and Klf7 showed a positive correlation with miRNAs expression levels, instead of the inverse correlation that would be expected if the miRNAs simply inhibited translation of their gene targets. These findings could be explained considering the miRNA – mRNA interactions constitute a complex network. Indeed, recent studies have suggested that miRNAs may act not only by merely reducing protein translation, but also through non conventional mechanisms (i.e. leading to increase in protein expression levels, binding target genes at 5'UTR region, guiding transcription factors to target promoter) (Cipolla 2014, Salmanidis 2014).

In conclusion, this work shows that miRNAs may contribute to the antidepressant mode of action through different patterns. The ability of miRNAs to regulate gene expression and, in particular, the fact that a given miRNA may regulate the expression of hundreds of target mRNAs, often associated in gene networks, makes them extremely attractive therapeutic drug targets for treating complex disorders, such as MD, where subtle changes to various components of a particular system may be effective. The different modes of gene expression regulation involving miRNAs suggest that additional work is needed in order to fully understand their exact role and to develop new avenues for therapeutic strategies.

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