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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular realizada sob a orientação científica do Professor Doutor Enrico Tongiorgi (Universidade de Trieste) e do Professor Doutor Carlos Duarte Bandeira (Universidade de Coimbra)

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## ABSTRACT

Depression is a mood disorder, which represents one of the major causes of disability of people worldwide. The major features of this disorder are the neuronal plasticity impairment, abnormal neuronal network and in some case a cell loss but the mechanism whereby they occur is still not fully understood. Antidepressant drugs, a common choice for depression treatment, target monoamine neurotransmitters systems decreasing serotonin and norepinephrine reuptake and consequently, promoting the restoring of the monoamine neurotransmission. Although a fast increase in monoamine levels with antidepressant administration, the therapeutic effects takes longer to be visible. Recent studies found that antidepressant drugs can increase the neurotrophin BDNF expression even before the rise of BDNF mRNA transcription, suggesting that they can potentiate postregulation of BDNF. These results have raised the working hypothesis of a role of miRNAs modulated by antidepressants in the BDNF gene regulation. With this purpose, we have evaluated the effect of miRNAs modulated by antidepressant treatment in the neuronal morphology and survival as well as their effect on BDNF expression.

Our results suggest that mir-302b, mir-214 and mir-136 do not cause morphological changes in neuronal morphology and survival since the up- or downregulation do not represented a differential morphological behavior. Measurements of BDNF expression levels modulating miRNAs were challenging; however, as many miRNAs are putative or validated targets for BDNF gene, mostly in the 3'UTR, it was evaluated the meaning of the 3' UTR long for neuronal cells. Truncation of the long 3' UTR BDNF led to a decrease of the total dendritic length at 6 and 12 DIV although the number of primary and dendrites remained unaltered.

Moreover, it was visible a lower number of mushroom spines at distal fields of matured neurons and a lower percentage of colocalization with PDS-95 and Syn I at 6, 9 and 12 DIV.

Taken together, the results shows that the absence of BDNF transcripts with the long 3'UTR ($BDNF^{lox}$ mice) do not have major impact on neuronal shape but rather a deficit in the total dendritic length and impairment of the synaptic maturation and connectivity. These evidences may be important to understand the role of the miRNAs on depression´s recovery. Since was not observed a neuronal morphological change acting on single miRNAs it may be possible that the BDNF translational regulation requires multiple miRNAs and translation factors acting in a cooperative but specific manner on the two populations of BDNF (BDNF 3'UTR short, BDNF 3'UTR long). This differential regulation can be underlined by detailed control of the synaptic maturation and activity in physiological and pathological conditions.

**Keywords:** Depression, Antidepressant drugs, miRNA, BDNF

A depressão é uma desordem psiquiátrica que representa uma das maiores causas de incapacidade em todo o Mundo, sendo que é caracterizada pelo enfraquecimento da plasticidade neuronal, anormal rede neuronal e, somente em alguns casos, perda celular. No entanto, o mecanismo pelo qual surgem estas alterações neuronais é ainda desconhecido. Os antidepressivos, largamente usados no tratamento de depressão têm como alvo os sistemas de transmissão de monoaminas, inibindo o reuptake destes neurotransmissores restabelecendo a transmissão monoamínica. Apesar de ser verificado um rápido aumento dos níveis de monoaminas com a administração de antidepressivos, o seu efeito terapêutico apenas se torna visível após um longo período. Estudos recentes demonstraram que os antidepressivos podem aumentar os níveis da neurotrofina BDNF antes de ser verificado um aumento do BDNF mRNA, o que sugere um efeito modulador mediado pelos antideprressivos na tradução de BDNF. Tais resultados originaram a hipótese de trabalho que se assenta na possibilidade de uma acção reguladora dos miRNAs na tradução do BDNF. Posto isto, teve-se como objectivo a avaliação do efeito de miRNAs modulados previamente por medicamentos antidepressivos na morfologia e sobrevivência neuronal, e igualmente na tradução de BDNF.

Os nossos resultados sugerem que os mir-302b, mir-214 e mir-136 não levam a alterações da morfologia e sobrevivência de neurónios do hipocampo com a sobre- ou subregulação destes miRNAs. A quantificação dos níveis de expressão de BDNF após modulação com os miRNAs mostrou-se de difícil execução; porém, tendo em conta que um número significativo de miRNAs é validado ou previsto para interagir com o gene

de BDNF, principalmente na 3' UTR, foi avaliado o significado funcional do 3' UTR nas células neuronais.

O truncamento da porção longa da 3' UTR (long 3' UTR) resultou numa diminuição do comprimento dendrítico total a 6 e 12 DIV embora nenhuma alteração tenha sido verificada no número de dendrites primárias e secundárias. Para além disso, foi visível uma diminuição do número de espículas "mushroom" em regiões distais de neurónios maduros e uma menor percentagem de colocalização entre PSD-95 e SynI a 6, 9 e 12 DIV.

Finalmente, os resultados obtidos mostram que a ausência de transcriptos BDNF com o "long 3'UTR" não têm um pronunciado efeito na forma dos neurónios mas são sim os responsáveis por um défice no comprimento dendrítico total, assim como na maturação sináptica e conectividade. Estas evidências podem ser importantes para entender o papel dos miRNAs na recuperação da depressão. Tendo em conta que não foram observadas alterações morfológicas com um único miRNA pode ser possível que a regulação da tradução de BDNF requira a acção de múltiplos miRNAs na regulação dos dois tipos de transcriptos de BDNF (short 3' UTR e long 3' UTR). Esta regulação diferencial pode ser analisada por um controlo detalhado da maturação sináptica e sua actividade em condições fisiológicas e patológicas.

**Palavras-chave:** Depressão, Antidepressivos, miRNAs, BDNF

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| | |
|---|---|
| **Ago** | argonaute protein |
| **AMPA** | α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid |
| **ANOVA** | Analysis of variance |
| **BDNF** | Brain-derived Neurotrophin Factor |
| **BSA** | Bovine serum albumin |
| **cAMP** | Cyclic adenosine 3‟,5‟-monophosphate |
| **CNS** | Central Nervous System |
| **CPE** | Carboxypeptidase |
| **CREB** | cAMP response element-binding protein |
| **DIV** | Days in vitro |
| **FGF** | Fibroblast Growth Factor |
| **FMRP** | Fragile X mental retardation protein |
| **HPA** | Hypothalamus-pituitary-adrenal axis |
| **LTD** | Long-term depression |
| **LTP** | Long-term Potentiation |
| **miR** | MicroRNA |
| **MMPs** | Metalloproteases |
| **MAPK** | Mitogen-activated Protein Kinase |
| **MAP2** | Microtubule-associated protein 2 |
| **NGF** | Nerve Growth factor |
| **NMDA** | N-methyl-D-aspartate |
| **NRI** | Norepinephrine reuptake inhibitor |
| **NTFs** | Neurotrophic Factors |

| | |
|---|---|
| **NT3** | Neurotrophin 3 |
| **NT4** | Neurotrophin 4 |
| **PCR** | Polymerase chain reaction |
| **PBS** | Phosphate buffered saline |
| **PLC-γ** | Phospholipase C-γ |
| **PI3K** | Phosphatidylinositol 3-Kinase |
| **PSD** | Postsynaptic density |
| **P75NTR** | p75 Neurotrophin Receptor |
| **RNA** | Ribonucleic acid |
| **RISC** | RNA-induced Silencing Complex |
| **SNRI** | Serotonin norepinephrine reuptake inhibitors |
| **SSRI** | Selective serotonin reuptake inhibitor |
| **TNF** | Tumour Necrosis Factor |
| **Trk** | Tropomyosin Receptor Kinase |
| **UTR** | Untranslated Region |

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## *Principles of neuronal plasticity*

Neuronal plasticity consists in the capacity of the Central Nervous System (CNS) to adapt to a changing environment through structural and functional rearrangement of neuronal connectivity. Neuronal plasticity is also a crucial element in injury recovery and in learning and memory (Pearson-Fuhrhop et al., 2009). This mechanism undergoes through generation of newborn neurons, degeneration of dispensable neurons and reorganization of synaptic connections. At cellular level, plasticity is determined by dendritic spines formation and their pruning and remodeling (Ethell et al., 2005), calcium channel regulation, alterations in NMDA receptors or in AMPA trafficking (Cull-Candy et al., 2004). Functionally, two well-known forms of plasticity are long-term potentiation (LTP) and long-term depression (LTD), the long lasting strengthening and weakening of synaptic connections between neurons.

Neuronal plasticity is initiated and maintained by synaptic activity, which in turn is stimulated by excitatory neurotransmitters, which cause an action potential on postsynaptic neurons and trigger transcription of genes involved in restructuring of CNS. Defects in this process will impair adaptation of the CNS to new challenges causing not only the disruption of homeostasis but increasing also the susceptibility of neuronal circuits to environmental variations. In this way, a failure to adapt may lead to structural and functional damages of specific brain regions, which in turn can cause progressive neurodegeneration triggering diverse pathologies.

***Neurotrophic factors as marker of plasticity***

Neurotrophic factors are growth factors that participate in the processes that guarantee neuronal survival. Neurons are dependent of these molecules for their survival and to the establishment of neuronal network and neuronal plasticity, including the modulation and remodeling of axonal and dendritic growth, membrane receptor trafficking, neurotransmitter release and formation of synapses. The neurotrophin family are constituted by four different proteins structurally related: nerve growth factor (NGF) (Levi-Montalcini et al., 1966), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) (Hohn et al., 1990). According to their affinity they can bind to two classes of membrane receptors: the tropomyosin receptor kinase (trk) family (Barbacid et al., 1994) and the p75 neurotrophin receptor (p75NTR), a member of tumour necrosis factor (TNF) receptor superfamily (Rodriguez-Tébar et al., 1990).

Neurotrophins are synthesized as precursor protein (pro-neurotrophins) that can be processed intracellularly, originating the mature and biologically active form of this protein, or instead be released as a pro-neurotrophin through regulated pattern (Mowla et al., 1999), modulating the development and maturation of neuronal circuits. Neurotrophins bind to trk receptors leading to their dimerization and autophosphorylation, allowing the alteration of their conformation to an activated state (Ullrich et al., 1990). Subsequently, trk receptors phosphorylate intracellular molecules activating signaling cascades. The effects mediated by mature neurotrophins are due to activation of high affinity full-length trk receptor, which activates pathways specifically involved in neuronal survival and growth. The four neurotrophins have diverse affinity to these receptors: NGF binds to trkA receptor, BDNF and NT-4 to trkB and NT-3

binds mostly to trkC but under some circumstances it may also bind to trkA or trkB (Barbacid et al., 1994; Cohen-Cory et al., 2010). In the other hand, pro-neurotrophins have high affinity to another type of receptors, the p75NTR receptor, which has low affinity to mature form of neurotrophin but high affinity for pro-neurotrophin, activating intracellular signaling pathways involved in cellular death (Lee et al., 2001; Dechant et al., 2002). Thus, mature and precursor form of neurotrophins and their binding to different receptors leads to divergent effects.

### *Brain-derived Neurotrophic Factor*

BDNF is the best well characterized neurotrophin and is currently seen as a factor with multiple and crucial functions. This neurotrophin was initially discovered through its ability to promote and support the survival of chick sensory neurons during development, being similar to fibroblast growth factor (FGF) (Barde et al., 1982). BDNF plays a key role in neuronal survival and maturation during development and neuronal plasticity in adulthood.

BDNF has specific mechanisms of transcription, translation and posttranslational modifications. BDNF gene has a complex and a well-conserved organization among mammals (Maisonpierre et al., 1991; Tettamanti et a., 2010), suggesting that regulation of its expression has a strong functional significance. Like the other neurotrophins, BDNF is synthesized as propeptide that may follow two different secretory pathways to either the regulated (i.e. release in response to stimuli) or constitutive pathway (i.e. spontaneous release). BDNF shows in majority of neurons a distal dendritic targeting of secretory granules through regulated pathway whereas constitutive pathway occurs mainly in the soma (Brigadski et al., 2005). Pro-BDNF is formed in the soma and can

be cleaved in the endoplasmic reticulum (Mowla et al., 2001) giving rise the mature neurotrophin or remaine in the unprocessed form. Pro-BDNF can be processed intracellularly by furin and proconvertases generating mature BDNF; otherwise, it can be cleaved extracellularly by metalloproteases (MMPs) such as plasmin (Lee et al., 2001; Teng et al., 2005). The targeting of BDNF for regulated or constitutive pathways is determined in Golgi complex through its binding to intracellular sortilin which promotes a correct folding of the mature domain (Teng et al., 2005; Lu et al., 2005). Subsequently, there is an interaction between sorting motif within mature domain and carboxypeptidase (CPE), which targets mature BDNF to regulated pathway (Lou et al., 2005). Mature BDNF can be packaged into synaptic vesicles present both in axon terminals (presynaptic site) and dendrites (postsynaptic site) of glutamatergic neurons and be released in activity-dependent manner through an influx of calcium (Wu et al., 2004; Matsuda et al., 2009).

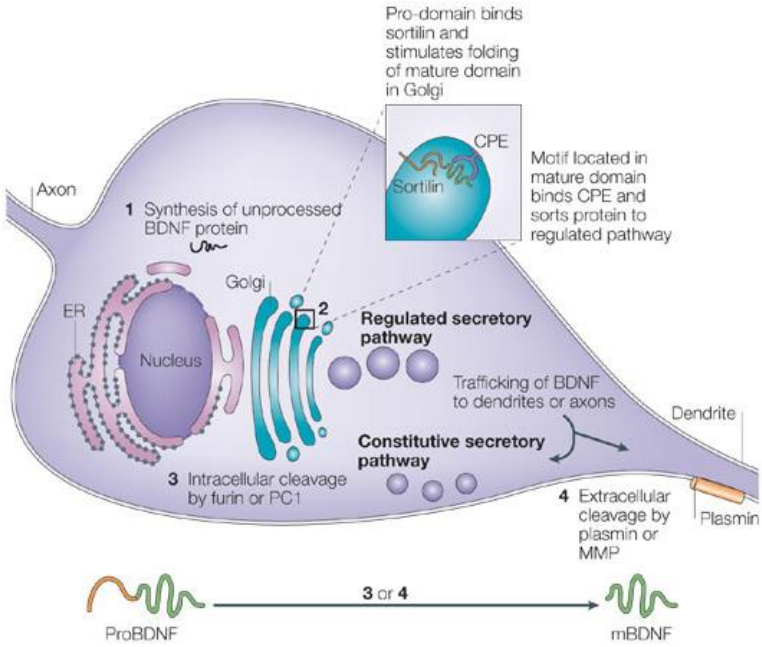


**Fig.1:** Synthesis and sorting of BDNF to either constitutive or regulated secretory pathway. Pro-BDNF is synthesized in the endoplasmic reticulum (ER) and interacts with sortilin in the Golgi complex what promotes the folding of the mature domain. Subsequently, mature domain can bind to carboxypeptidase E (CPE) sorting mBDNF to secretory vesicles of the regulated pathway, which transport BDNF to dendrites, or axon to its release; if this interaction doesn´t happens BDNF is sorted to constitutive pathway. Instead of mature BDNF even proBDNF can be released by neurons if the proneurotrophin isn´t cleaved by furin or protein convertases (as protein convertase 1- PC1). In these cases, proBDNF can be cleaved extracellularly by metalloproteinases (MMP) and plasmin (Bai Lu; 2005).

As mature and precursor BDNF activate trkB receptor or p75NTR, respectively, different effects can be achieved. Stimulation of the trkB receptor stimulation activates patterns involved in cell survival (such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and phospholipase C-y˝ (PLC- y˝) pathways) while p75NTR stimulation leads to apoptosis (activating caspase cascades, jun kinase and also p53 pathways) (Fišar et al., 2010).

The BDNF gene consists in multiple alternative 5´untranslated exons in different number according to the species (ten in humans, eight in rodents and six or even less in other vertebrates) each of them under individual promoter regions, and a single exon coding for the entire BDNF pre-protein amino acid sequence. The transcription of BDNF exons are achieved by individual promoters differentially regulated taking into account the developmental stage, target tissue and neuronal activity (Aid et al., 2007). The regulation of promoters by a wide range of stimuli and their activation by neuronal activity and DNA methylation enables the expression and target of their transcripts to different brain regions, different cell types and even different regions at the same cell (soma vs. dendrites) ensuring the precise spatial and temporal BDNF expression (Timmusk et al., 1993; Tongiorgi et al., 2006). Besides transcriptional regulation also BDNF translation through regulatory elements serve as another control mechanism to avoid translation of BDNF when is not needed (Croll et al., 1999; Cunha et al., 2009).

In addition to the possible BDNF isoforms obtained by transcription of different 5'UTR exons through differential activity of promoters, additional complexity is provided due to the presence of alternative sites of polyadenilation in 3'UTR which give rise to two populations of BDNF transcripts: with a short 3´untranslated region (UTR) or with a long 3´untranslated region (Fig.2) (Timmusk et al., 1993).

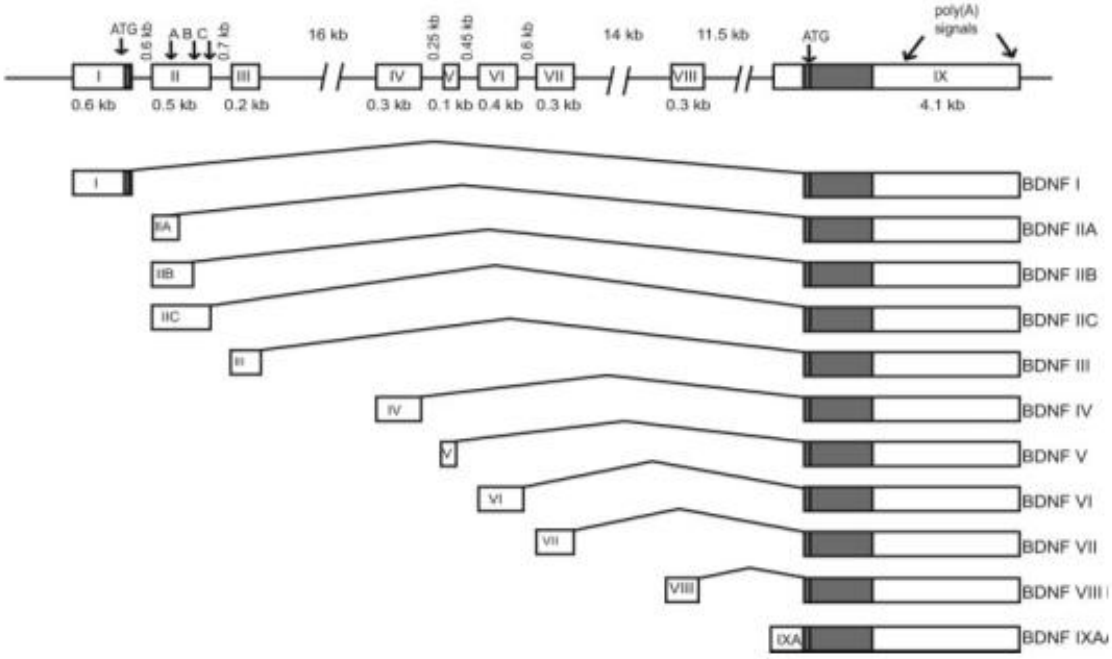


**Fig.2:** Scheme of rat BDNF gene structure and its transcripts. The eight non-coding exons of rat are spliced with the 3' exon-coding for BDNF protein (exon IX), originating different BDNF isoforms. Moreover, transcription can be initiated befor exon IX what leads to the IXA transcripts. In addition, transcripts can be polyadenilated at two polyadenilation sites (represented as arrows), what creates three transcripts variants for the exon II (IIA, IIB and IIC). Lines represent introns, boxes the exons and protein-coding exons are represented as solid boxes. (Aid et al., 2007).

The existence of these two pools of transcripts gave rise to the debate of their functional meaning in neuronal population. To study the role of these two types of transcripts were generated a specific mice strain, truncated for long 3'UTR, namely BDNFlox mice (Gorski et al., 2003). This mouse is created using a cre-site-specific DNA recombinase system in which lox sites are inserted into exon 5 (the protein-coding exon) of BDNF, upstream of lox sites a trimerized polyadenilation signal (Simian Virus 40 (SV40)) and downstream an Escherichia coli lac Z gene. Following the lac Z gene there is an Flp recombinase target (FRT)-flanked phosphoglycerate kinase (PGK)

neomycin cassette. The Cre-mediated recombinase deletes the polyadenilation signals abolishing the long portion of the 3'UTR of BDNF (Gorski et al, 2003).

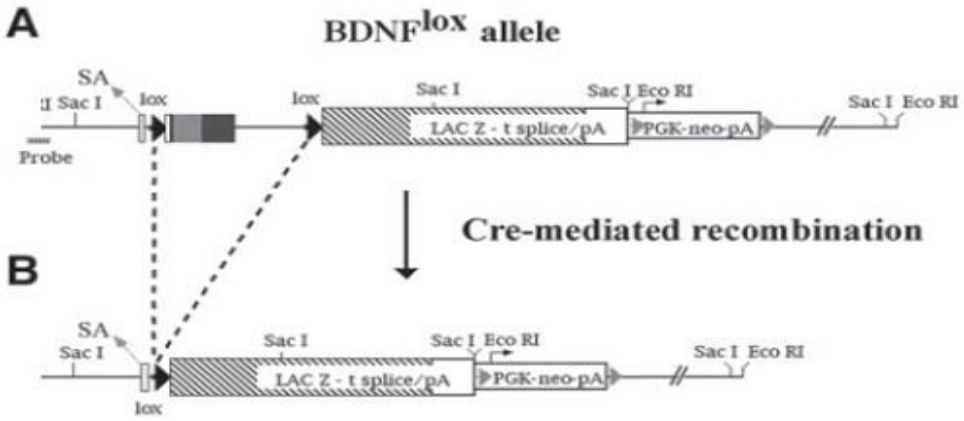


**Fig.3:** Cre-site-specific DNA recombinase system used to generate BDNFlox mice. Exon V, the BDNF coding exon, is shown schematically at the top of the figure in which the open bar identifies the 5'UTR; the shaded bar the pro domain of the BDNF; the filled bar the mature hormone region, flanked by lox sites (the filled triangles), and followed by lacZ, a SV40 intron-polyA (open bar) and a FRT-flanked PGK neomycin cassete. Location of the splice acceptor (SA) is denoted by an arrow. After Cre-mediated recombination BDNF-coding sequences in exon V are excised and lacZ is brought downstream of the splice acceptor site and under control of BDNF promoters (Gorski et al, 2003).

So far, had been suggested that transcripts with short 3'UTR are restricted to the soma while transcripts with long 3'UTR can be present in the soma or targeted to dendrites (An et al., 2008). However, two another studies showed that the two populations of transcripts are capable of being targeted to dendrites but in a differents extent (Yoneda et al., 2010; Baj et al., 2011) since long 3'UTR BDNF transcripts can reach more distal dendritic fields in comparison with short 3'UTR BDNF transcripts.

The knowledge of subcellular localization is important to understand the contribution of each type of transcript to local changes in neuronal structure and function.

### *BDNF implication in brain function*

BDNF is the most abundant growth factor in the brain and is essential for maintenance of neuronal function and neurogenesis. In cases in which BDNF levels are low the brain shows structural abnormalities and reduced neuronal plasticity. The level of BDNF should be strictly regulated to permit a balance between the enhancing of axon arbor growth and its connectivity with its targets and the destabilization and pruning of axon terminals. A reduced BDNF level induces presynapric site destabilization and axon pruning (Hu et al., 2005) what results in a smaller number of presynaptic terminals and excitatory synapses (Luikart et al., 2005). Thus, BDNF is not only necessary to initiate axon branch but also to the stability of the axon arbor already formed as well as the synaptic connections.

The development of growth axon cone is possible due to axon guidance molecules that activate intracellular signaling pathways responsible for the growth of filopodia and lamellipodia. However, BDNF in neuronal cultures also stimulates filopodia and lamellipodia dynamics along the axon by inducing localized changes in the actin cytoskeleton (Gibney et al., 2003; Yuan et al., 2003). Observations made in vivo had shown that axon guidance and pathfinding are independent of BDNF action as demonstrated by normal guidance of major afferent projections in trkB mutant mice. Nevertheless, events that follow successful axon pathfinding depend on BDNF otherwise synaptic connectivity of axon with targets is affected (Rico et al., 2002; Huang et al., 2007).

In addition, BDNF has a crucial role in the modulation of the number of synaptic vesicles docked in the active zone of presynaptic terminals, which is probably proportional to neurotransmitter release. The amount of docked vesicles can be altered upon disturbance of BDNF signaling (Shen et al., 2006). Indeed, deficits in trk signaling results in the decrease of the expression of synaptic proteins involved in synaptic vesicle docking and fusion, decreasing the number of docked vesicles at active zones and, consequently, downregulating neurotransmitter release (Lin et al., 2000). On the contrary, exposure to BDNF increases the number of synaptic vesicles docked at active zone in excitatory synapses in hippocampal neuronal cultures (Tyler et al., 2001). Nevertheless, BDNF also acts under inhibitory synapses inducing glutamic acid decarboxylase (GAD) expression, explaining the reason why alterations in trk signaling and reduced levels of BDNF leads to a decrease in the number of GABAergic boutons and synaptic specialization (Rico et al., 2002; Kohara et al., 2007). Thereby, BDNF has a crucial role in balancing the number of excitatory and inhibitory synapses in adult brain.

***Dendritic spines as vital component of synaptic activity***

Most of synaptic activity takes place in dendritic spines, a dendritic protusion that contains a scaffold rich in elements of the biochemical signaling machinery. These structures mediate the excitatory neurotransmission in the brain and are present as thousands on pyramidal cells.

Spines are dynamic structures that can present a wide range of sizes and shapes during their lifetime. Generally, spines are morphological characterized as structures with a bulbous head connected to dendritic shaft by a narrow neck (Sorra and Harris,

2000). The spine head contains the postsynaptic density, constituted by ion channels and cell surface receptors in scaffold with cytoplasmic scaffolding and signaling proteins (Li and Sheng, 2003), which permit the link between the postsynaptic density to actin filaments, the main cytoskeleton component of dendritic spines (Dent et al., 2011). Dendritic spines are mostly represented by three types of spines: "mushroom-like" spines which have a big round head and a tiny spine neck that makes the connection between the spine and dendrite; "stubby" spines which are short and without a defined spine neck and "thin" spines which length is bigger than the neck which in turn is similar to spine head (Jen claude Béique 2012). The spine development and maturation is regulated in a synapse-specific manner (Jen claude Béique 2012). Development of dendritic spines will promote the morphological and biochemical changes in a manner that allows the passage from stubby to thin spines and finally mushroom spines, the most mature spine type (Grutzender et al., 2002). Spine formation is regulated by different components. One of them is BDNF which contributes to spine formation, promoting the reshuffle of early filopodia-mediated contacts (Matsutani et al., 2004). The promotion of spine maturation can be evaluated through the amount of postsynaptic specializations, which is higher or lower depending of the increase or decrease of BDNF levels (Sanchez et al., 2006).

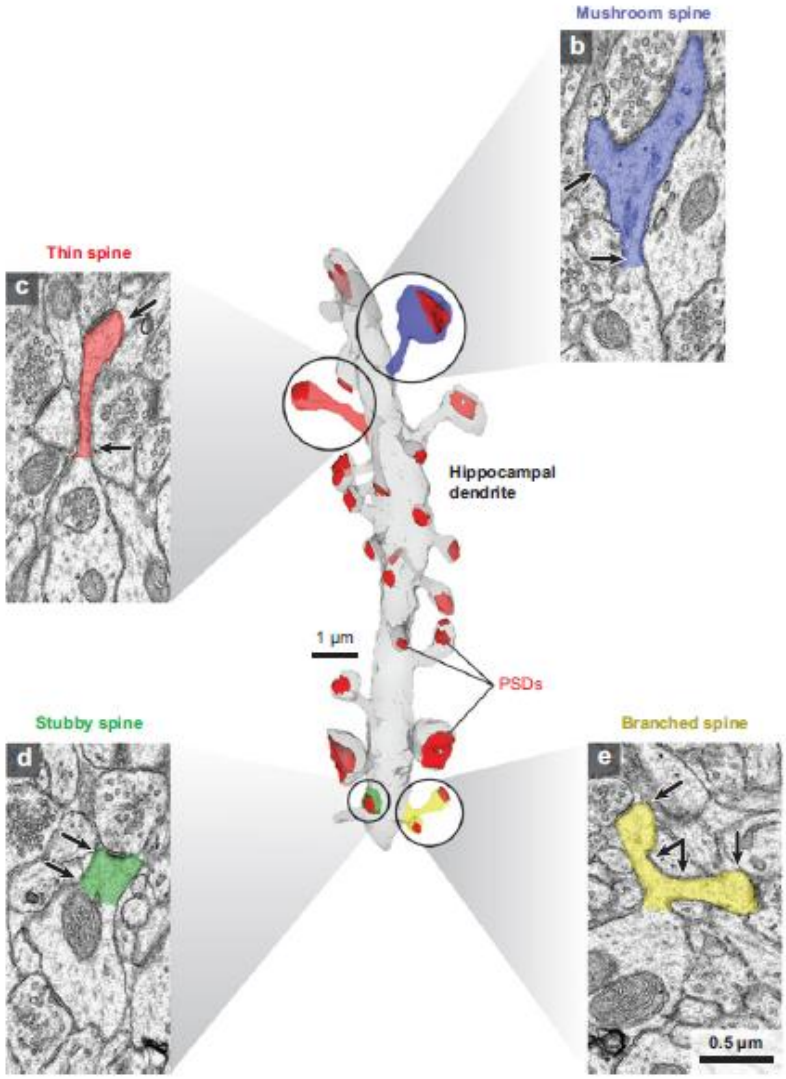


**Fig.4:** Three-dimension illustration of different types of spines of a neuronal dendritic segment. Mushroom spines have a big spine head and a small neck; "stubby" spines are characterized by short and without a defined spine neck; "thin" spines are long spines with a small head and branched spines are like two thin spines (Harris et al., 2008).

## *Depression: a worldwide disorder*

Depression is a common mood disorder affecting 121 million people worldwide (Mouillet-Richard et al., 2012) among children, adolescents, adults and elderly and according to World Health Organization it is predicted to be between of the top three of

causes of disability by 2030. The symptoms of this stress-related disorder manifest as changes in cognitive functions, learning, memory and emotions (Lipsky et al, 2002) which seem to be caused by neuronal plasticity impairment, abnormal neuronal network and in some cases atrophy or even cell loss produced by stress events, characteristics of low adaptation capacity of CNS to the environment (Fišar et al., 2009; Drzyzga et al., 2009).

An interesting point that drew attention of many researchers was the higher predisposition of certain individuals to develop depression which could indicate that, at least in part, depression is genetically-determined (Levinson 2005). Stressful events, defined as alterations of the homeostasis, are in fact a risk factor for depression. Despite genetically-determined susceptibility-threshold specific for each individual to variations in brain environment it is also accepted that stress events occurred in critical period of brain development can increase the vulnerability to stress and favour the onset of anxiety disorders (Muiños-Gimeno et al., 2011). However, it is important to notice that depending on the type of stress event and the affected brain regions the consequences in the brain function can be completely different. A soft stress in the hippocampus increases learning and memory processes (Luine et al., 1996) whereas severe stress has the opposite effects, impairing Long-Term Potentiation (LTP) (Kim and Diamond, 2002). Moreover, different brain areas have diverse pathways to respond to stimuli explaining why continuous stress in the hippocampus and prefrontal cortex leads to neuronal atrophy while in amygdale it causes neuronal hypertrophy (Sapolsky, 2003). Nevertheless, independently of stress rate and its effect in neuronal network, seems to be a common characteristic the long lasting effects on vulnerable individuals (Roth et al., 2009).

Moreover, depressive patients present a reduction of the hippocampus volume in about 4-5% when compared with healthy individuals, which is correlated with disease duration and consequent dysfunction (Sheline et al., 2003).

Activation of different patterns by stressors can explain the duration of the effects mentioned above. One of the possible mechanisms consists in the dysfunction of the hypothalamus-pituitary-adrenal (HPA) axis. Disturbance of this axis and alteration of glucocorticoids receptors expression and signaling leads to the lack on the recognition of glucocorticoids levels, which decrease the negative feedback and consequently increase the level of glucocorticoids in circulation and the response to stressors, explaining the long-lasting effects of stress events (Min et al., 2012). Other possible mechanism is related to the impairment of neuronal plasticity and lower monoamine neurotransmission (Massart et al., 2012). Different studies performed with depressed patients' brains show brain structural alterations, decrease on the expression of neuronal markers as well as alterations in neurotrophins expression (Fišar et al., 2009). In this point, neurotrophic factors (NTFs) can play an important role and be a connection between stress and psychiatric disorders.

The summary of results obtained until now allowed the stipulation of two different but potentially complementary hypotheses that can be implicated in the origin of this depression. The first one, the monoamine hypothesis, poses that depression is a result of the deficit in serotonin and noradrenaline neurotransmission while the neurotrophic hypothesis suggests that depression is caused by the diminished levels of neurotrophins, more specifically, BDNF.

BDNF has been seen as a component that takes part in disease pathologies or treatment of many psychiatric diseases. The mechanism whereby psychiatric disorders

occur is not fully understood so far, however BDNF seems to be involved in the pathophysiology and therapeutic mechanisms of depression (Fuchikami et al 2010).

### *Antidepressant drugs*

Antidepressant drugs have been used for the past 50 years but the exact mechanism of their action is not fully understood. These drugs include the selective serotonin reuptake inhibitors (SSRIs) (Cramer et al, 2009), norepinephrine reuptake inhibitors (NRIs) or inhibitors of both as serotonin norepinephrine reuptake inhibitors (SNRIs) (Kellermann et al., 2012) which target monoamine neurotransmitters systems in order to increase signaling at the synapses. Their therapeutic effect passes through the increase of monoamine neurotransmitter to restore the compromised neurotransmission in noradrenergic and serotorinergic system. However, their effects are only visible when administrated for long periods, taking about 6-8 weeks to produce therapeutic effects, despite of the increased synaptic monoamine level hours after antidepressant treatment, demonstrating that the recovery of this synaptic transmission cannot be the only outcome produced by antidepressants to explain the extent of their clinical effects (Yu et al., 2011). The delayed efficacy of antidepressants could be due to a requirement of neuroadaptative mechanisms that may increase neuronal plasticity. Chronic antidepressant administration of both 5-HT and norepinephrine selective inhibitors increases cAMP-CREB signaling that will increase BDNF expression (Duman and Monteggia et al., 2006; Nibuya et al., 1996), restoring its normal level and neuronal plasticity. Furthermore, the stimulus caused by the antidepressants can also have an impact in dendritic growth and arbor complexity (Magill et al., 2010).

Thus, antidepressants can stimulate through the mosulation of neurotrophic factors the development of neuronal morphology and synaptic plasticity that is important for the behavioral effects of the treatment.

### *Effects of antidepressant treatment on BDNF level*

Antidepressants are capable of modulate BDNF expression provoking its increase in situations in which its expression is abnormally low, restoring defective signaling systems, enhancing the neuronal plasticity, supporting synaptic connectivity and replacing neuronal cytoarchitecture (Calabrese et al., 2009, Castren et al., 2007, Russo-Neudstadt and Chen, 2005). These drugs increase mature BDNF form level in synaptic compartments and restore trkB signaling (Calabrese et al., 2007), suggesting that BDNF can be a huge effector of their mechanism. This increase in mBDNF levels could be due to an increase of local translation of BDNF transcripts or to the trafficking of BDNF protein from other compartments (Molteni et al., 2009; Tongiorgi et al., 2008).

The consequences on BDNF expression will depend on the time of exposure to antidepressant treatment. Chronic treatments increase BDNF expression (Xu et al., 2004; Tsankova et al., 2006) while acute treatment have no effect or can even reduce total BDNF mRNA (Drzyzga et al., 2009). Moreover, different classes of antidepressants act differentially on BDNF transcription recruiting different BDNF promoters and even reverting epigenetic modifications caused by stress events (Yu et al., 2011).

For antidepressants-mediated behavioral effects TrkB signaling is essential (Saarelainen et al., 2003). Antidepressants can cause a higher phosphorylation of trkB receptor increasing its signaling and at the same time increasing the phosphorylation of

CREB (Castren et al., 2009), a mediator of BDNF gene transcription that will result in the increase of BDNF expression and trkB activation. Therefore, neuronal plasticity and cellular functions are restored.

It is thought that one of the main mechanisms through which BDNF contributes to antidepressant effect is its ability to promote translation of BDNF.

### *Dysregulation of microRNAs in depression*

The recent discovery that BDNF can exert its control on protein translation through regulation of miRNAs has opened completely new perspectives for the role of neurotrophic factors and miRNAs.

Gene transcription and translation is highly regulated by different mechanisms being one of them the regulation mediated by miRNAs (miRs). MiRNAs are a class of small non-coding RNAs with a length of about 22 nucleotides that participate in fundamental intracellular mechanism involved in gene expression regulation (Yiing et al., 2006). More than 400 miRNAs have already been identified in human and chimpanzee brains (Bushati et al., 2008) but is estimated that more than 1000 miRNAs could be expressed in human brain. Despite of many miRNAs being conserved between species, some miRNAs seem to be specific to human, suggesting a recent origin (Bushati 2008). In the genome, miRs can be located in introns of protein-coding and non-coding genes, exons of non-coding genes or in intragenic regions (Kim et al., 2005).

Alterations in miRNAs system have been associated to generation of several diseases such Alzheimer (Satoh et al., 2010), Parkinson (Mena et al., 2010),

schizophrenia (Lai et al., 2011) and depression (Saus et al., 2010) indicating that miRNAs play a critical role in the enhanced susceptibility for many disorders.

### *Biogenesis of miRNAs*

MiRNAs are derived from precursor molecules, the primary miRNA (pri-miRNA), that is originated through transcription of miRNA genes by RNA polymerase II and III. After transcription there is a processing of pri-miRNA in the nucleus by the enzyme Drosha that has like cofactor the DGCR8. This process generates small hairpins precursors, the pre-miRNAs, which normally have about 70-110 nucleotides and are folded into stem-loop structure. In some cases, a small group of intronic miRNAs, called mitrons, is generated from hairpin introns. Their processing don´t involve the enzyme Drosha since pre-miRNA is generated through introns by nuclear splicing machinery. Formed pre-miRNAs are transported via exportin transfer system to cytoplasm where takes place the processing of this molecules by RNA enzyme Dicer forming the double-stranded mature small RNA (miRNA/miRNA* duplexes). One the molecule of miRNA/miRNA* duplexes is loaded onto an Argonaute homologue protein leading to the formation of RNA-induced silencing complex (RISC), whereas the other miRNA* strand is degraded (Dwivedi et al.,2011).

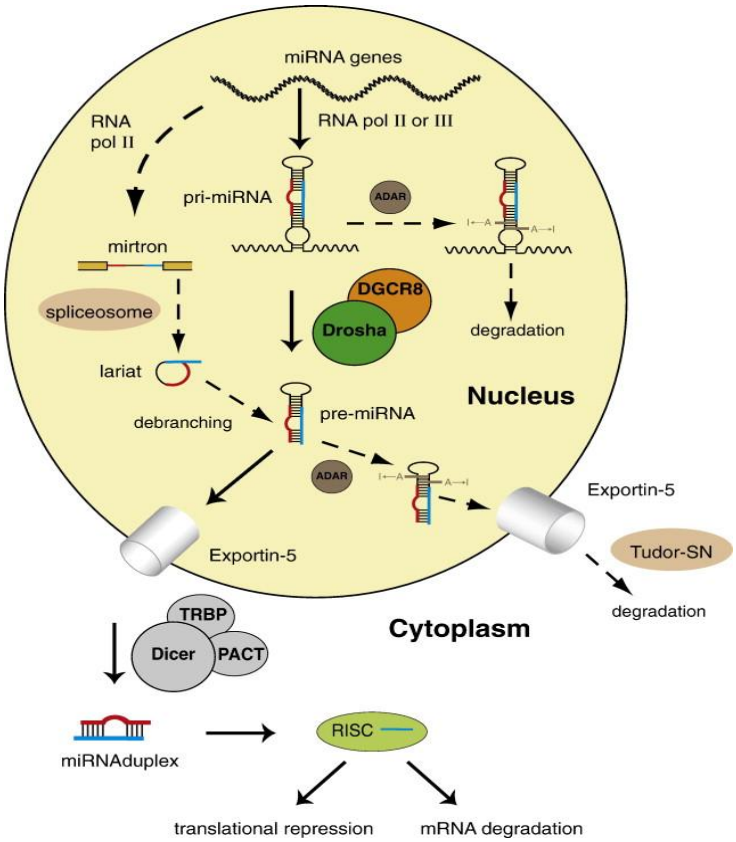


**Fig.5:** Ilustration of the synthesis mechanism of miRNAs. miRNA genes are transcribed by RNA polymerase II and III originating pri-miRNAs molecules that is processed in the nucleus through the enzyme Drosha, generating the pre-miRNAs. Occasionally, intronic miRNAs (mitrons) can be generated from hairpin introns. Pre-miRNAs are transported to cytoplasm via exportin where are processed by RNA enzyme Dicer forming the double-stranded mature small RNA that posteriorly forms the RISC complex (Faller et al., 2008).

### *The role of miRNAs in brain function*

MiRNAs have the capacity of binding to a target site, generally within 3' untranslated region (3'UTR) of an mRNA via base-pairing interactions, inducing their degradation or inhibition of translation (Mellios et al., 2008). The inhibitory action of miRNAs doesn´t requires a perfect complementary between miRNA-mRNA (Le et al., 2009); what determines miRNA target specificity seems to be the "seed sequence" of the miRNA that is constituted by 6-7 nucleotides (Bushati et al., 2008). Nevertheless,

miRNA-mediated regulation is not only due to translation inhibition but may in some circumstances act as activators of translation (Krol et al, 2010) or even impair transcription through their binding to gene promoters (Younger and Corey, 2011).

MiRNAs act as a control of protein synthesis and are considered as mediators of mRNA turnover and even as a mechanism by which can be determined the amount of expressed protein (Calissano et al., 2010). These molecules can effectively regulate the amount and timing of the protein expressed and determines the localization of protein synthesis. This feature is extremely important to the correct function of the neuronal network taking into account that mRNAs are synthesized in the nucleus while gene products are often required at the distal ends of dendrites and axons, far away from the local of synthesis (Abdelmohsen et al., 2010). The translation of mRNAs stays inhibited until neurons stimulation. The regulation of local translation of mRNAs seems to have a crucial role in synaptic development and plasticity (Schratt et al., 2006).

In higher eukaryotes, miRNAs are involved in diverse mechanism of cellular and organ physiology such as development, differentiation and diseases (Calissano et al., 2010), being important genomic regulators. Their expression is temporally and/or spatially-dependent during development (Bushati et al., 2008) becoming vital for development of nervous system and brain morphology and more specific in neuronal differentiation, integrity and plasticity (such as mir-124a and mir-133b), formation of synapses in postmitotic neurons (mir-134 and mir-9a) and also circadian rythms (mir-132 and mir-219) (Bushati et al., 2008; Abdelmohsen et al., 2010; Mellios et al., 2008). Indeed, during development the differential expression and activity of miRNAs influence the differentiation of different cells in the organism.

Although the ablations of certain miRNAs doesn´t cause, necessarily, the total loss of proper development, it can however lead to significant abnormalities (Andersson

et al., 2010; Abdelmohsen et al., 2010). In fact, the level of expression of miRNAs, their tissue distribution and the developmental stage in which they are expressed influence severally the expression of the target gene products (Abdelmohsen et al., 2010). Defects on miRNA system result in a decrease of dendritic complexity, altered spine morphology and cognitive defects showing that miRNAs are essential for normal brain development and establishment of functional brain connectivity (Magill et al., 2010).

The consequence of the interaction miRNA-mRNA is determined by a member of the argonaute (Ago) protein family, a catalytic component of the RISC complex. Depending of the type of argonaute protein and the degree of complementary between miRNA and its target sequence, the targeted mRNA may be endonucleolytical cleaved and degraded or translational repressed. The repression of the translation might be done by deccaping and subsequent exonucleotic degradation (Bushati et al., 2008). One single miRNA can target hundreds of mRNAs regulating the expression of several transcripts. Therefore, if one miRNA is present at abnormal levels the expression of several genes could be severely disturbed (Bushati et al., 2008). This disruption is correlated to many diseases since regulation of gene expression is an important aspect influencing neuronal plasticity. Moreover, the expression of miRNAs is a critical point for the survival of specific types of mature neurons being neuroprotector, preventing or slowing the neuronal loss (Bushati et al., 2008).

### *Impact of miRNAs in neuronal cells*

External signals have a significant impact in morphology and function of neurons. Such impact can be mediated by posttranscriptional modifications, a vital mechanism that controls locally the plasticity of dendrites, axons and synapses. It is likely that some

miRNAs can bind to mRNAs coding for neuron-specific proteins, regulating mechanisms as synaptic plasticity, neurogenesis, neurite outgrowth and stress responses (Abdelmohsen et al., 2010, Dwivedi et al., 2011). Interestingly, turnover of miRNA seems to be faster in comparison with other cells what can be a sign of the rapid adaptation to neuronal activity mediated by miRNAs (Krol et al., 2010)

MiRNAs play an important role in synaptic plasticity regulation. They are expressed within dendrites regulating the expression of a variety of synaptic mRNAs that mediate dendritic growth and keep mRNAs in a dormant state while are transported within dendrites to synaptic sites. In the absence of an external stimulus, miRNAs inhibit the expression of synaptic proteins controlling the spine development. When trk receptor is activated through BDNF the signaling pathway can inactivate the miRNAs leading to an enhanced expression of synaptic proteins and, consequently, spine growth (Schratt et al., 2006).

The way by which miRNAS are dendritically localized is not yet understood. A possible explanation is that miRNAs in its mature form can arrive in dendrites by passive diffusion or can be even possible that pre-miRs are not completely processed in cell body but rather appear to be transported to dendrites where are targeted into dendritic spines through an interaction with postsynaptic proteins. At this point, DICER and RISC components are responsible for processing pre-miRs originating mature miRNAs in an activity-dependent manner. MiRNAs are then responsible for local regulation of protein translation (Smalheiser et al., 2009).

Studies in animal models of depression and in human postmortem brain provided evidences for the role of miRNAs in psychiatric disorders since their expression appear altered in depressive and bipolar patients. Instead, psychoactive drugs are capable of

revert the miRNAs expression to normal levels even in brain and blood suggesting that their action could be mediated by miRNAs.

### *BDNF regulation mediated by miRNAs*

Pyramidal neurons, the primary source of BDNF in cerebral cortex, show a high expression of DICER, a needful enzyme for miRNA biogenesis, as well as RISC components. As BDNF exhibits multiple target sites for miRNAs, especially in 3´UTR, they can target this neurotrophin reducing its expression (Mellios et al., 2008). This feature points to a potential role for miRNAs to differentially regulate BDNF expression during development. As previously demonstrated overexpression of a specific miRNA can target BDNF and nullify its capacity to stimulate neurite growth. MiRNAs can interact at posttranscriptional level of BDNF and interfere with the control of neuronal morphology and function (Abdelmohsen et al., 2010).

The expression regulation of BDNF in dendrites mediated by miRNAs may serve as a mechanism to regulate BDNF function in space and time controlling the maintenance of dendritic arbor structure or acting as modifying factor to the shape and number of dendritic spines. For this reason is important to know exactly how and which miRNAs act under BDNF to understand the mechanism of mood disorders and the mechanism of action of antidepressant drugs achieving a better therapeutic for these disorders.

Depression is a mood disorder affecting a crescent number of people worldwide. The study of its mechanisms is mandatorily for the therapy of this disorder, representing a field of large interest. Recent studies found that different antidepressant drugs can activate translation of BDNF protein before the actual rise in the expression of its coding mRNA in hippocampus, suggesting that antidepressants may rapidly affect posttranscriptional regulation of BDNF (Musazzi et al, 2009). This lack of correspondence between BDNF mRNA and protein following antidepressant treatments (Jacobsen and Mork, 2004) suggests that antidepressants may have a particularly fast effect on posttranscriptional regulation of BDNF. These preliminary results suggest the working hypothesis of a role for miRNAs regulation of BDNF in the action of antidepressants. For these reasons, this study has the following objectives:

**1) To evaluate the role of miRNAs up- or downregulated by antidepressant drugs in neuronal morphology, cellular survival and BDNF translation:**

Treatment of adult rats during 2 weeks with antidepressant drugs manufacture by Servier (experiments performed by Prof. Maurizio Popoli's lab at the University of Milan) have resulted in the up- or down-regulation of a list of miRNAs. From these miRNAs was chosen a group of miRNAs from which was assessed their role in neuronal survival and development in vitro of primary cultures of rat hippocampal neurons through a morphological assay. Moreover, another goal is the evaluation of miRNAs effects on BDNF translation. In this way, we pretend to support the potential role of miRNAs in the mechanism of action of the antidepressant drugs.

**2) To evaluate the functional meaning of 3'UTR long of BDNF mRNA in the developmental neuronal morphology:**

As miRNAs target preferentially the 3'UTR of mRNAs it may be possible the differential regulation of the two populations of BDNF transcripts, either with long or short 3'UTR, by miRNAs. A previous study in our lab showed that the two populations of transcripts are present in the soma and also capable of being targeted to dendrites but in different extents, being possible to detect long 3'UTR BDNF transcripts in more distal dendritic fields in comparison with 3'UTR short BDNF transcripts (Baj et al., 2011). For this reason, it is expected that transcripts and consequently its proteins, depending of the 3'UTR, play different roles in neurons. According to these observations we had as an objective of this project the evaluation of the role of 3'UTR long BDNF transcripts in neuronal and spine morphology through neuronal hippocampal neurons from $BDNF^{lox}$ mice, mice truncated for 3'UTR long of BDNF.

## 3.1. Cellular Biology

***Culture of hippocampal neurons***

Cultures of hippocampal neurons were prepared from postnatal day 1-2 (P1-2) of Wistar rat or BDNFlox mice pups. The preparation of the 24-multiwell plates (Sarstedt), containing 12mm² coverslips (Sacco) especially for immunofluorescence experiments were coated with poly-L-ornithine 100 μg/mL for 20 min, washed once with PBS (phosphate buffer solution containing 137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.76mM KH2PO4, pH 7.4) and treated with 0,2% Matrigel ™ (BD Bioscience).

To extraction of hippocampi from Wistar rat hippocampi were dissected and kept in 3mL of cold Hank's balanced salts solution (NaHCO3 4.2 mM, Hank's salt powder 0.952%, HEPES 12mM (4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid), D-Glucose 33mM, kinurenic acid 100mM, penicillin 1 mg/mL (Euroclone), and streptomycin 1 mg/mL (Euroclone); pH 7.4). The dissected tissue was then digested with 500 μL of 0.25% trypsin in native MEM (Minimum Essential Medium + Earle's salts + GlutaMAX™; Euroclone), for 8 min at 37° C. Digestion was blocked with 5.5 mL of Dulbecco´s modified eagle´s medium (D-MEM- Euroclone) supplemented with 10% heat inactivated Fetal Bovine Serum (H.I. FBS, Gibco) and 1% *penicillin*/*streptomycin* (Euroclone *EC B3001D* 100x) of penicillin-streptomycin (Euroclone) and centrifuged at 900 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in supplemented D-MEM. In order to dissociate in single cells, the resuspended pellet was mechanically dissociated and

slowly filtered with a sterile 40 µm pore diameter strainer (Sarstedt), removing impurities and cellular debris. Finally, cell resuspension was plated onto 24-well plates at a cell density of 150-200.000 cells/well.

To extract hippocampi from BDNFlox mice, was used the procedure previously described. The BDNFlox transgenic mice model was kindly provided from Kevin R. Jones laboratory and knockout mice for the long 3'UTR (BDNFlox) were obtained by crossing heterozygous mice. In this case the hippocampi coming from each mouse were dissected and plated separately in order to isolate knockout, wild type and heterozygous mice neurons. Both hippocampi of each animal were dissected and kept in 0.5mL of cold (4ºC) Halk's balanced salts solution and digested with 175 µL of 0.25% trypsin in native MEM for 8 min at 37° C. Digestion was blocked with 2 mL of D-MEM supplemented with 10% heat inactivated Fetal Bovine Serum (H.I. FBS, Gibco) and 1% *penicillin*/*streptomycin* (Euroclone *EC B3001D* 100x) and the dissected tissue was then centrifuged at 900 rpm for 5 min at room temperature. The pellet was resuspended with supplemented D-MEM and filtered through a sterile 40µm pore diameter strainer (Sarstedt). Finally, cell resuspension was plated onto 24-well plates achieving a cell density of 90.000-130.000 cells/well.

Cells were maintained in vitro at 37 °C in a 5% CO2-humified incubator. Cell number was assessed by a dye exclusion method using Trypan Blue (Fluka). After 1h of incubation to permit a proper adhesion, medium was replaced by fresh Neurobasal™ Medium (Gibco) supplemented with 2% B-27 supplement (Invitrogen), 0.5% of 1mM L-glutamine and 10% penicillin 100U/mL and Streptomycin 100U/mL allowing long term growth and viability of postnatal hippocampal neurons and at the same time avoiding glia proliferation. Non-neuronal cells on rat hippocampal cultures were further

prevented by adding 5.0µM cytosine b-D-arabinofuranoside (ARA-C) from the second day in culture.

***Neuronal transfection with plasmids***

Neurons were transfected with GFP plasmids at 5, 8 and 10 DIV using Lipofectamine™ 2000 (Invitrogen) and OptiMEM (Gibco). The transfection mix was first prepared separately: one part containing 2µg of GFP and OptiMEM and the other with 2µl of Lipofectamine and OptiMEM, in both cases performing a total volume of 50µl. Each component was gently mixed and incubated at room temperature for 5 min. After that they were mixed and incubated for further 20min at RT and in the meanwhile the culture medium was replaced for 500 µl new fresh supplemented Neurobasal medium. Cells were incubated with the transfection mix (100µl for each coverslip) for 1 hour at 37° C in a 5% CO2-humified incubator. After 1h of incubation the medium was once again replaced for new fresh supplemented Neurobasal and the cells were incubated at 37°C until immunofluorescence experiments were performed.

After 24h (6 and 9 DIV) or 48h (12 DIV) of transfection with GFP, hippocampal neurons were blocked. The culture medium was removed and was done a PBS wash, followed by fixation with 4% paraformaldehyde (dissolved in PBS pH 7.2) for 20 min and a final wash in PBS.

***Neuronal transfection with synthetic oligonucleotides***

To transfect neurons with BLOCK-iTTM Alexa Fluor® Red Fluorescent Control (Invitrogen) the transfection was performed at 5 DIV using Lipofectamine™ 2000

(Invitrogen) and OptiMEM (Gibco). The transfection mix was first prepared separately: one part containing the required concentration of Block-it and OptiMEM and the other with 2µl of transfection reagent (either Lipofectamine™ 2000 or HiPerFect (Qiagen)), 2µg of GFP and OptiMEM, in both cases performing a total volume of 50µl. In other hand, to transfect neurons with miRNAs mimics or inhibitors, both synthesized by Qiagen, the transfection was performed at 9 DIV using Lipofectamine™ 2000 (Invitrogen) and OptiMEM (Gibco). The transfection mix was first prepared separately: one part containing 50nM of miRNA mimics or 100nm of miRNA inhibitors and OptiMEM and the other with 2µl of Lipofectamine, 2µg of GFP and OptiMEM, in both cases performing a total volume of 50µl.

Each component was gently mixed and incubated at room temperature for 5 min. After that they were mixed and incubated for further 20min at RT and in the meanwhile the culture medium was replaced for 500 µl new fresh supplemented Neurobasal. Cells were incubated with the transfection mix (100µl for each coverslip) for 1 hour at 37° C in a 5% CO2-humified incubator. After 1h of incubation the medium was once again replaced for new fresh supplemented Neurobasal and the cells were incubated at 37°C until immunofluorescence experiments were performed.

After 24h of neurons transfection with Block-it hippocampal and 72h after miRNAs mimics/inhibitors transfection neurons were blocked. The culture medium was removed and was done a PBS wash, followed by fixation with 4% paraformaldehyde (dissolved in PBS) for 20 min and a final wash in PBS.

***PC12 cell line experiments***

Rat PC12 pheochromocytoma cells (ATCC, Manassas, VA, USA) were seeded in 25cm2 flasks (Sarstedt) in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies) supplemented with 10% of heat-inactivated horse serum (HS) and 5% of fetal bovine serum (FBS). The culture was incubated at 37° C in a 5% CO2-humified incubator and the medium was renovated every 2-3 days with fresh RPMI supplemented medium. When confluence was achieved cells were passage 1:3.

For cell viability purpose, cells were replaced into a 15mL falcon and centrifuged at 900 rpm for 5min at RT and the pellet was resuspended in low-serum RPMI medium constituted by 5% of HS and 2% of FBS in order to promote cell attachment. Cells were then seeded in 96-well plate previously covered with poly-L-ornithine 100 µg/mL for 20 min and 0,2% Matrigel ™ (BD Bioscience) in a cell density of 1x106 cells/well (Han et al., 2012). Cell number was assessed by a dye exclusion method using Trypan Blue (Fluka). Transfection was performed 1 day after plating. The 96-well plate was divided in two groups, one transfected at 72h and the other at 48h before doing MTT cell viability experiments. Each group contains cells transfected with the miRNAs mimics, miRNAs inhibitors, cells treated only with Lipofectamine ™ 2000 (Invitrogen) and untreated cells, all conditions in quadruplicate. The transfection mix was first prepared separately: one part containing 50nM of miRNA mimics or 100nm of miRNA inhibitors and serum-free RPMI medium and the other with 0,4µl of Lipofectamine, 2µg of GFP and serum-free RPMI medium, in both cases performing a total volume of 20µl. Each component was gently mixed and incubated at room temperature for 5 min. After that they were mixed and incubated for further 20min at RT. Cells were incubated

with the transfection mix (20µl for each coverslip) for 72 and 48 hours at 37° C in a 5% CO2-humified incubator.

***MTT assay viability***

After incubation of PC12 with miRNAs mimics or inhibitors the cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, MTT (Sigma) was dissolved in PBS 1X to make a 5mg/mL solution and 10 µl of this solution was added to each well of the 96-well flat bottom plate (Sarstedt) and incubated at 37 ◦C in a 5% CO2-humified incubator for 4h. Finished the incubation time with MTT the supernatant was carefully removed and the formed formazan crystals were dissolved in 100 µl of isopropanol-0,1HCl solution. The plates were then analyzed using a Microplate Reader at 560 nm and 600nm. Data was collected from two independent experiments.

## 3.2. Molecular Biology

***GFP plasmids preparation***

In order to prepare GFP plasmids to further cellular transfection, was performed a MiniPrep with pGFP-N1 plasmids (Clonetech). Firstly the bacterias were placed into an Agar plus ampicilin petri-dish and were incubated at 37ºC O/N. On the day after, were picked two of the existing ampicilin-resistent colonies placing them into 5mL of LB medium and incubating O/N at 37°C with shaker 200 rpm. The LB medium was then

divided in two 50mL falcons that are subject to miniprep purification. For plasmid purification were used the kit of Promega. Cells were centrifuged at 5000g for 10min at RT, supernatant were discarded and the pellet resuspended in 3mL of cell resuspension solution. Cell lysis solution was added and mixed gently being incubated for 3min. To the lisate cells were added neutralization solution that was centrifuge at 15000g for 15min which originates a pellet of cellular debris that can be discarded. The supernatant, with remaining debris, were cleaned through PureYieldTM Clearing Columns and a vacuum manifold. PureYieldTM Binding Column was used in order to promote the binding of DNA to its membrane. The membrane was washed several times and DNA was eluted in 500µl of miliQ water.

***Genotyping of BDNF$^{lox}$ mice***

The genotypes of BDNFlox mice were identified by PCR. For the extraction of genomic DNA from tails each mouse tail was incubated with 300 µL of Extraction Buffer (Tris-HCl 10mM pH 8.0, SDS 0.5%, EDTA 0.1M pH 8.0, RNAse 0.2%) at 37 °C for 1 hour, followed by incubation with 2 µL of Proteinase K (15 mg/mL) at 50 °C O/N. On the second day was added 300 µl of Phenol-Chloroform:Isoamyl alcohol (pH 8.0) to each sample to remove the proteins from the nucleic acids, the samples were homogenized in the shaker for 20 minutes. The samples were centrifuged at 12.000 rpm for 10 minutes in order to separate the nucleic acids (upper phase) from the denaturated proteins (interphase) and the lipids (lower phase). The aqueous phase, containing the genomic DNA, was removed to another eppendorf, diluted with 2 volumes of Ethanol 100%, thoroughly mixed and stored at -80°C for 30 minutes to enable DNA precipitation. Finally, all samples were centrifuged at 4°C for 10 minutes at 12.000 rpm

and the DNA pellet was resuspended in 100 µl of DNAse - free water. To confirm the extraction of genomic DNA was ran in 10% agarose gel at 80V.

The genotypes were assessed by PCR with specific primers to amplify either the long and short 3´UTR of BDNF (reverse primer 5'- ATT ACA AGC AGA TGG GCC AC-3'; forward primer 5'- ACC TGG GTA GGA CAA GTT G-3' (Eurofins MWG Operon)). The PCR reactions were performed in a final volume of 20 µl containing 0,1µL Go Taq Polymerase (Promega Corporation), 4 µl 5X Green Go Taq Buffer , 0,4 µl dNTPs, 0,8µl MgCl2, 1 µl of each primer, 11,7 µl of DNAse - free water and 1 µl of cDNA added as PCR template. The PCR conditions were set as follow: 5 minutes at 95°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C; 10 min at 72°C. The PCR generates a 250bp product for short 3´UTR and 230bp products for long 3´UTR BDNF observed in 15% agarose gel.

***Immunocytochemistry for MAP2, SynI and PSD-95***

Immunocytochemistry was done in the coverslips previously used for cultures of hippocampal neurons at 6, 9 and 12 DIV. The cells were permeabilized with PBS-Tryton 0,1% for 15 minutes at RT. After permeabilization the coverslips were incubated with mix of primary antibody (table 1) containing either 10% of NGS and PBS-Tryton 0,1% to perform the total volume (70µl for each coverslip) for 1,5 hour at room temperature. Cells were then washed several times in PBS in order to remove the unbound antibody and were posteriorly incubated for 1h at RT with the secondary antibody (table 3). After the incubation the coverslips were once again washed in PBS and underwent to a final incubation with Höechst dye solution (0,001%) in order to stain all cellular nuclei. Finally, the coverslips were washed with PBS and Milli-Q

water and mounted on a glass slide using anti-fade mountant containing Mowiol® 40-88 (Sigma) to be further analyzed at the fluorescence microscope.

**Table 1:** Primary and secondary antibodies for immunocytochemistry for MAP2, SynI and PS95

| Primary antibody | Dilution | Host | Supplier |
|---|---|---|---|
| MAP2 | 1:200 | Rabbit polyclonal | Santa Cruz Biotechnology |
| PSD95 | 1:250 | Mouse monoclonal | Millipore |
| Syn I | 1:200 | Rabbit polyclonal | Millipore |

***Immunocytochemistry for miRNAs experiments***

Immunocytochemistry was done in the coverslips used for cultures of hippocampal neuron previously transfected with miRNAs mimics and inhibitors at 12 DIV. The permeabilization and blocking of the cells was done with 10% NGS, 1% BSA and 0,3M glycine- PBS-Tween 0,1% for 45 minutes at RT. After permeabilization was done a sequential immunocytochemistry in which coverslips were immuno-stained with the primary antibody for BDNF (Sigma) O/N at RT followed by the incubation with the primary antibody for the miRNA targets (diluted in PBS-Tryton 0,1%) (Table 2) for 2 hours at RT (for FMRP, PTEN, MeCP2) or O/N at 4ºC (for LIMK1). Cells were then washed several times in PBS in order to remove the unbound antibody and were posteriorly incubated for 1h at RT with the secondary antibody (table 3). After the incubation the coverslips were washed with PBS, and underwent to a final incubation with Höechst dye solution (0,001%) in order to stain all cellular nuclei. Finally, coverslips were washed with PBS and Milli-Q water and mounted on a glass slide using

anti-fade mountant containing Mowiol® 40-88 (Sigma) to be further analyzed at the fluorescence microscope.

**Table 2:** Primary and secondary antibodies for immunocytochemistry of miRNAs experiments

| miRNA | Primary antibody (αmiRNAs target) | Dilution | Host | Supplier |
|---|---|---|---|---|
| mir-134 | LIMK1 | 1,5µg/ml | Rabbit polyclonal | Abcam |
| mir-302b | FMRP | 1:400 | Rabbit polyclonal | Abcam |
| mir-214<br>mir-136 | PTEN | 1:100 | Rabbit polyclonal | Abcam |
| mir-132 | MeCP2 | 1:150 | Mouse monoclonal | Genescript |

**Table 3:** Secondary antibodies for immunocytochemistry experiments

| Secondary antibody | Dilution | Fluorophores | Supplier |
|---|---|---|---|
| Anti-Rabbit | 1:200 | Alexa 568 | Invitrogen |
| Anti-Mouse | 1:200 | Alexa 568 | Invitrogen |
| Anti-mouse | 1:200 | Alexa 647 | Invitrogen |

## 3.3. Imaging and Analysis

***Fluorescence microscopy***

Digital images of GFP-positive neurons either with MAP2 staining or immunoreactive for miRNAs targets and at the same time with a nuclear Höechst staining were acquired using a Nikon Eclipse E800 epifluorescence microscopy with a 20X objective and a Nikon DXM1200 camera, paired with ACT-1 software. The Höechst staining was visualized with a 488nm fluororophe while MAP2 and miRNAs targets were observed with 568nm fluorophore. The pictures were taken in blind.

***Confocal microscopy***

Images to spines measurements and densytometric analysis were acquired through Nikon C1si confocal microscope, containing 457, 477, 488, 514 argon lasers lines and 561 or 640 nm diode laser. Light was delivered to the sample with an 80/20 reflector. The system was operated with a pinhole size of Airy disk (30 nm) (for spines images the pinhole was set 60 nm). Electronic zoom was kept at minimum values for measurements to reduce potential bleaching. A 60X Oil Apo objective (with corresponding NA of 1.4) was used to collect series of optical images at 0.15 μm (for spine images) or 0.20 μm (for densitometry) z resolution step size with a corresponding voxel size of 75x75x150 nm (X x Y x Z). Images were then processed for z-projection by using ImageJ 1.45 (NIH, Bethesda, USA).

Spines images were collected as 40μm of both proximal and distal fields from the neuronal apical dendrite, classification based in well defined parameters (table 4).

**Table 4: Parameters defining proximal and distal fields of neurons**

| | Proximal field | Distal Field |
|---|---|---|
| Diameter (μm) | ≥ 2 | ≤ 2 |
| Distance from the soma (μm) | ≤ 60 | ≥ 70 |

The set of parameters to acquire the confocal pictures for densytometric analysis were maintained exactly the same between pictures from either hippocampal neurons under miRNAs mimics and inhibitors action and control.

***Cellular viability through Höechst staining***

To determine cell density all 12mm$^2$ glass coverslips (Sacco) used to neuronal culture had been visually divided in four squared fields being collected one picture on each one and one more in the periphery of the cross between two fields (Fig.6). With this approach the count was done in a reproducible and reliable way. Using the multipoint tool of Imagej was possible to count the number of glia cells and neurons present in each field taking into account that neurons nuclei have a smaller dimension and a stronger and brighter intensity on Höechst staining when compared with glia cells.

To determine the effect of the treatment with miRNAs mimics/inhibitors in cell viability was done the average of the cell density of 3 independent experiments comparing each treatment condition with control.

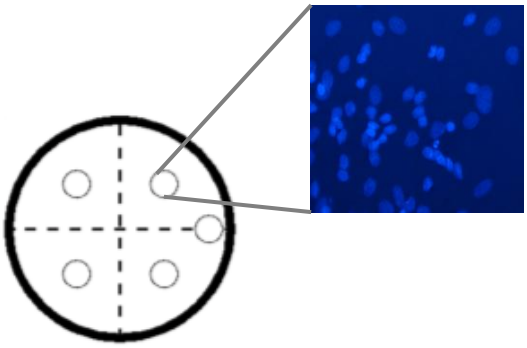

**Fig.6:** Approach to collect pictures from the coverslips used for cultures of hippocampal neurons in order to measure cellular viability via Höechst staining. A picture of nuclei demonstrates the differences in the size and immunoreactive intensity between neurons and glia cells.

### *Neuronal morphological analysis*

The morphological analysis of hippocampal neurons was focused on four different parameters: average of total dendritic length, number of primary dendrites, number of secondary dendrites and number of branching points. To reach this aim, the morphologic parameters of GFP-positive neurons were measured and quantified by tracing all neuronal projections using ImageJ software (NIH, Bethesda, USA).

In our analysis was measured the length of every dendritic arbors in separately. A dendritic arbor was consider as a protrusion emerging from the cell soma with all its branches and, so, primary and secondary dendrites. The sum of the dendritic arbors length gave us the total dendritic length of the measured neuron. The branching points were counted using the Multi-point tool of Imagej. Filopodia protrusions (considered as >5 microns-long) were excluded from the total branching point measurements.

In order to achieve a stronger statistic analysis were measured 44-65 neurons for each genotype and time point in the case of BDNFlox mice experiments and about 30 neurons for each miRNA mimic/ inhibitor. The measurements were done in blind.

***Polarization analysis***

To define the degree of polarization of the neuronal culture we have used the method previously described by Horton and Ehlers (Horton and Ehlers, 2006). Briefly, was evaluated the contribution of the length of the individual dendrites (Lm) of each neuron, considered as the primary dendrite with all its branches, for the total dendritic length. In this way, was done the average of the length of the individual dendrites (Lsym) and posteriorly was performed the calculations in the following manner:

Lm / Lsym = Length of dendrite / Total dendritic length

If dendrites were symmetric, the value of the proportion above would be equal: Lm =Lsym =1/number of dendrites (Lm/Lsym = 1).

If dendrites were not symmetric the ratio Lm/Lsym would reflect the degree to which dendrites diverge from perfect symmetry (Lm/Lsym = 1). All dendrites that presented an Lm/Lsym value above the threshold fixed at 2 have been considered as "polarized" dendrites. Finally we have calculated the percentage of neurons that have a polarized dendrite, meaning that are polarized neurons. As an alternate measure of dendritic polarity we determined the fractional contribution of the primary dendrite (1°), of the next longest dendrite (2◦) as well as of the 3° longest and so on until the smallest (7°).

### *Spine morphological analysis*

Dendritic spines have been classified as Stubby, Thin and Mushroom based on the morphological criteria proposed by Harris et al. (Harris et al. 1992) (Figure 2). More specific, are defined as "Stubby" the spines in which neck diameter and spine total length are comparable; "Thin" if the length of the spine is much greater than both neck and head diameters that are both similar and "Mushroom" if the spines have a big head diameter and a small neck. Following a visual classification based on known morphological criteria, the straight line tool of ImageJ software was used to measure the spine length, traced from the edge of the dendrite to its farthest point, the spine head diameter as well as the dendrite length (Fig.14). The spine density was reached through the quotient between the number of spines found in the proximal or distal field and the dendritic length (40µm).

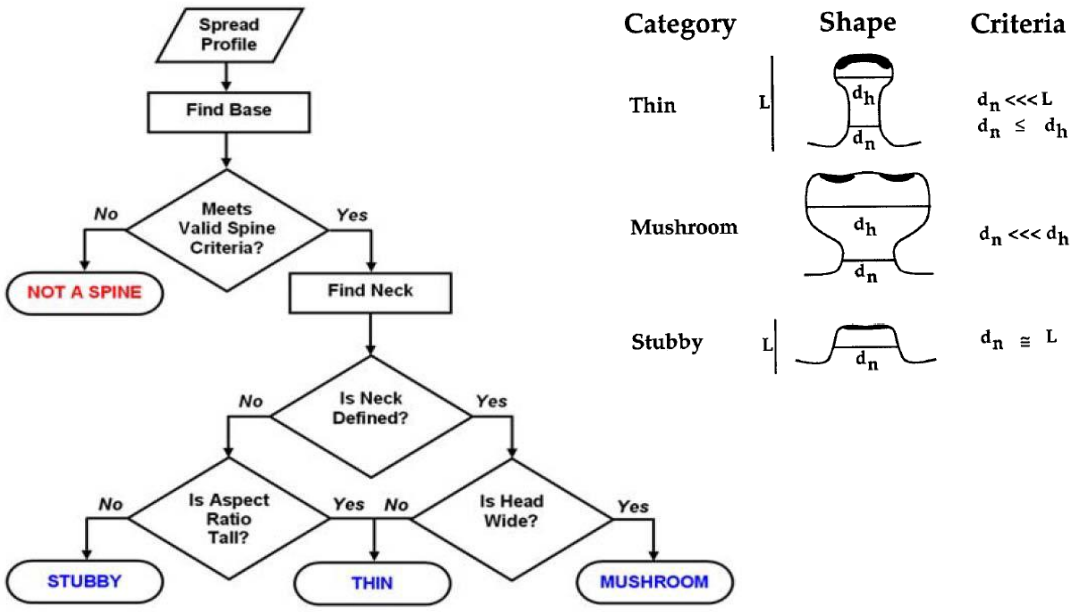


**Fig.7:** Criteria taking into account for spines classification performed by the operator. "Stubby" spines have neck diameter and spine total length comparable; "Thin" if the length of the spine is much greater than both neck and head diameters that are both similar and "Mushroom" if the spines have a big head diameter and a small neck. (Harris et al., 1992; Rodriguez et al., 2008).

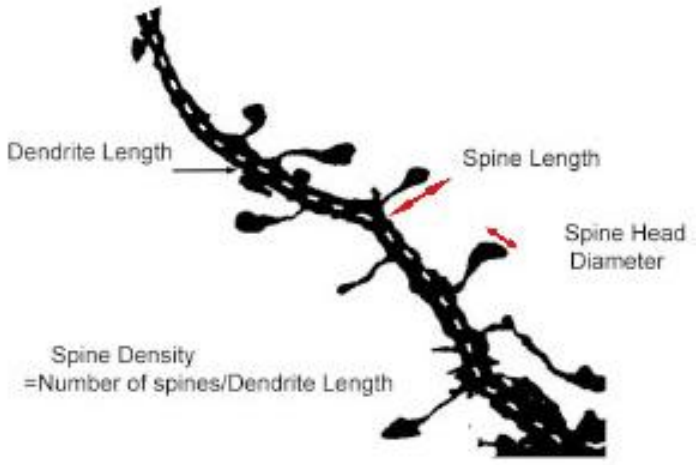


**Fig.8:** Approach to measurement of spines in order to do a good analysis of spine morphology between different conditions of development of the culture of hippocampal neurons (Smith et al. 2009).

### *Co-localization of Syn-I and PSD-95*

The degree of Synapsin-1 and PSD-95 co-localization had been evaluated starting from confocal images of both proximal and distal fields of the apical dendrites. In particular Alexa-586-labeled secondary antibody (bound to Synapsin-1 primary antibody) was visualized using a 561 nm diode laser while PDS-95 signal was detected by excitation of the Alexa-647 secondary antibody through a 640 nm diode laser. Moreover, to avoid any possible cross-talk phenomena among fluorophores, all images were collected using the method of sequential line scanning. The final quantification of colocalized pixel was performed using the software Imaris (Bitplane) and its colocalization module. The software was used with default setting with the addition of an automatic threshold for pixel intensity calculated on real point spread function for our microscopy and optics. The colocalization was quantified as amount of fluorescence collected from each pixel stained (over the threshold) for both protein targets.

***Bioinformatic analysis of miRNAs targets***

The search of targets for miRNAs was performed with miRWalk database that provides information on the predicted and validated targets and their binding sites in human, rat and mouse organisms. The predicted targets are achieved through the study of the possible interactions between miRNAs and target genes whereas the validated targets module provide information about experimentally verified miRNA interaction, both of them presenting the interaction sites in complete sequence (promoter, 5' UTR, CDS and 3' UTR) of all known genes. These two modules have their last update on March 2011, crucial for reliable results. The information of the possible interactions is gave by 9 different algorithms (miRWalk, Diana-microT, miRanda, miRDB, PICTAR, PITA, RNA22, RNAhybrid and Targetscan) which present for each miRNA or gene target a range information such as: number of algorithms that recognize a specific complementary between miRNA-target, P-value, number of nucleotides of miRNA seed sequence, position of the binding site, between others.

Finished the analysis of the validated targets for the miRNAs (mir-132, mir-134, mir-302b, mir-214), among the total number of gene targets they were subject to a selection taking into account important aspects such as the presence of the target protein in neurons and implication in crucial pathways of neuron development. For the analysis of the predicted miRNAs that have as target BDNF, among the wide range of miRs, were selected only the miRs that make part of the group that are in agreement with the following parameters: recognition of the interaction miRNA-BDNF for at least 2 algorithms, miRNA seed sequence constituted for 8 nucleotides minimum, binding-site in the 3´UTR of BDNF transcript and a validated target expressed in neurons.

### *Densitometric analysis*

Evaluation of the effect of miRNAs mimic or inhibitors on the expression level of the targeted protein was performed on confocal pictures densitometric analysis. Within experiments all conditions, including illumination intensity and photomultiplier gains were kept constant. For proteins that are only expressed in the soma the densitometric analysis focused precisely in this area while with proteins expressed in the total neurons were also analyzed secondary branching points. The densitometry of the positive-signal for the target protein was achieved through Measure Stack plugin of ImageJ Software and the chosen neuron area to further analysis were every time the same between neurons in the same picture. The densitometry of each neuron is reached through the sum of the immunoreactivity of all neuron stacks. As densitometric value for each treatment, to further comparison between miRNAs mimics and inhibitors effects on neurons, was calculated the mean fluorescence intensity for each group. To quantify experimental data obtained from separate days, data were normalized relative to mean fluorescence intensity in control neurons. Moreover, were also normalized the collected data with mean fluorescence intensity of backgroung in each picture. To achieve a strong statistic meaning were analyzed 25 neurons for each miRNA mimics, inhibitors and controls.

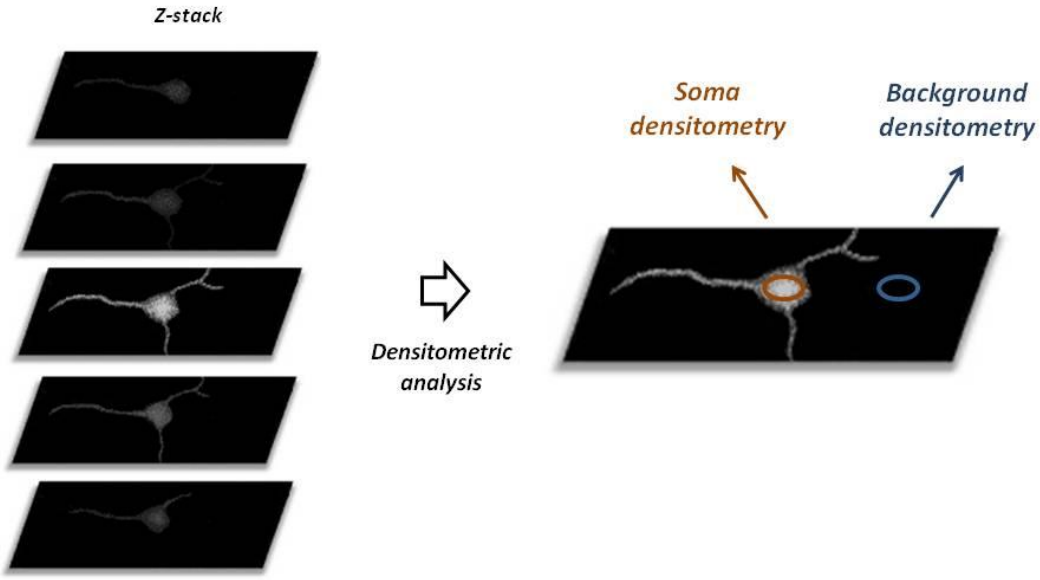


**Fig.9:** Densitometric analysis to quantification of protein expression level. Using ImageJ Software the densitometry of a specific area of the neuron is measure and normalized with the background.

### *Data representation and statistic analysis*

The results were represented as mean of all measurements with corresponding standard errors (SE). All graphs and statistical analysis were performed with Sigma Plot 11 Software (Systat Software, Inc).

Statistical significance among groups was evaluated performing both t-test, and One Way ANOVA followed by an all pairwise multiple comparison procedure with Holm-Sidak's method where ***, $P \leq 0.001$, **, $P \leq 0.01$, and, *, $P \leq 0.5$.

## 4.1. Role of miRNAs up- or downregulated by antidepressant drugs in neuronal morphology, survival and BDNF translation

Recently, a growing number of evidence point for the involvement of miRNAs in the mechanism of antidepressant drugs (Zhou et al., 2009; Baudry et al., 2010; Angelucci et al., 2011; He et al.; 2012; Smalheiser et al., 2012). To evaluate the effects of antidepressant treatment on miRNAs expression, adult rats were treated with different antidepressant drugs (agomelatine, fluoxetine and desipramine) during two weeks. The RNA extraction from hippocampus of rats treated with antidepressants or untreated was processed and analyzed using microarrays technology to evaluate the miRNAs level expression. Further statistical analysis showed significant alteration of the expression level of certain miRNAs upon treatment. Antidepressant treatment and microarrays experiments were performed by our collaborators Prof. Maurizio Popoli´s lab at the University of Milan (www.unimi.it). From the list of miRNAs that were altered upon treatment we have chosen three that presented the higher or the lowest fold change when compared with control levels obtained from untreated rats to further screening in vitro (Table 5).

**Table 5: MiRNAs compiled from microarray analysis from RNA of adult rats treated with different antidepressant drugs.**

| miRNA | Antidepressant drug | Fold Change |
|---|---|---|
| mmu-mir-136 | Agomelatine | 0.48 |
| mmu-mir-302b | Fluoxetine | 3.11 |
| | Desipramine | 3.47 |
| hsa-mir-214 | Fluoxetine | 0.44 |
| | Desipramine | 0.56 |

To evaluate miRNAs effects on neuronal morphology, survival and BDNF translation the required techniques were performed on postnatal hippocampal neurons from rat Wistar. In order to address these goals the neurons were treated either with miRNAs mimics or miRNAs inhibitors oligonucleotides (Qiagen) so as to acquired similar conditions to those obtained with up- or downregulation of endogenous miRNAs and elucidate the biological role of the miRNAs on neuronal function.

***Optimization of neuronal transfection with synthetic oligonucleotides***

In the absence of a well establish protocol to neuronal transfection with synthetic oligonucleotides, it was necessary to set up the exact conditions to do it, not only in a way that guarantees the neuronal transfection with miRs but also to ensure that they are actually acting and producing a similar decrease or increase on gene expression.

Since miRs mimics/inhibitors oligonucleotides are not fluorescently labeled, being impossible to know their exact localization into the culture and verify the neuronal

transfection with these oligonucleotides, was used the BLOCK-iT (Invitrogen), a red-labeled dsRNA oligomer with similar length, charge and configuration to miRs. The sequence of BLOCK-iT is not homologous to any known gene avoiding the possibility of BLOCK-iT-mediated effects by silencing neuronal proteins, serving as a control of transfection efficiency facilitating assessment and optimization of oligonucleotides delivery into cells. In this way, the transfection of hippocampal neurons at DIV 5 and DIV 11 was done with different concentrations of BLOCK-iT (5nM, 25nM, 50nM and 100nM) using either Lipofectamine (Invitrogen) transfection reagent or Hiperfect (Qiagen), a transfection reagent especially design to delivery siRNA or miRNA. Additionally, hippocampal neurons were also co-transfected with GFP plasmids to allow the visualization of the entire cell morphology in green to further morphological analysis. The transfected coverslips were fixed 24h after transfection using PFA 4% and were immuno-stained using antibody against MAP2 proteins and even with Hoechst staining for cellular nuclei (see methods). All images were obtained by choosing 5 fields of the coverslips as described at methods (see Fig.6) using the 20X objective of epifluorescence microscope. The transfection efficiency was obtained through the ratio between BLOCK-iT-positive cells (nuclear red-labeled) and the total number of neurons in culture. This measurement was performed using multipoint tool of Imagej Software making the discrimination between neurons and glia cells (see methods, Fig.6).

BLOCK-iT-positive neurons were present in a really small number when transfected with the lower concentration of BLOCK-iT, recommended by the supplier, being reached less than 0,1% with Hiperfect transfectant and about 2% with Lipofectamine2000 of tranfection efficiency. The transfection efficiency started to be higher from 25nM of BLOCK-iT; nevertheless, this increase was merely observed with Lipofectamine2000 reagent rather than Hiperfect, which seems not to work properly.

For this reason, transfection with the higher concentrations of BLOCK-iT (50nM and 100nM) was just performed with Lipofectamine reagent. Surprisingly, transfection efficiency for these concentrations reached extremely high values: 88,9% and 96,7% of neurons are positive for BLOCK-it when it´s used 50nM or 100nM of this oligonucleotide, respectively. Simultaneously, the cell density upon transfection with BLOCK-iT was measured in order to verify if transfection with such high concentration of synthetic oligonucleotide is toxic to neuronal cells. To measure the cell density images of 5 fields of the coverslips were taken as described at methods (see Fig.6) using the 20X objective of the epifluorescence microscope followed by the count of the cell density of two different cultures. Cell density of all experiments did not suffer significant changes when compared with untreated neurons, which means that transfection with BLOCK-iT doesn´t have a toxic effect (Fig,10).

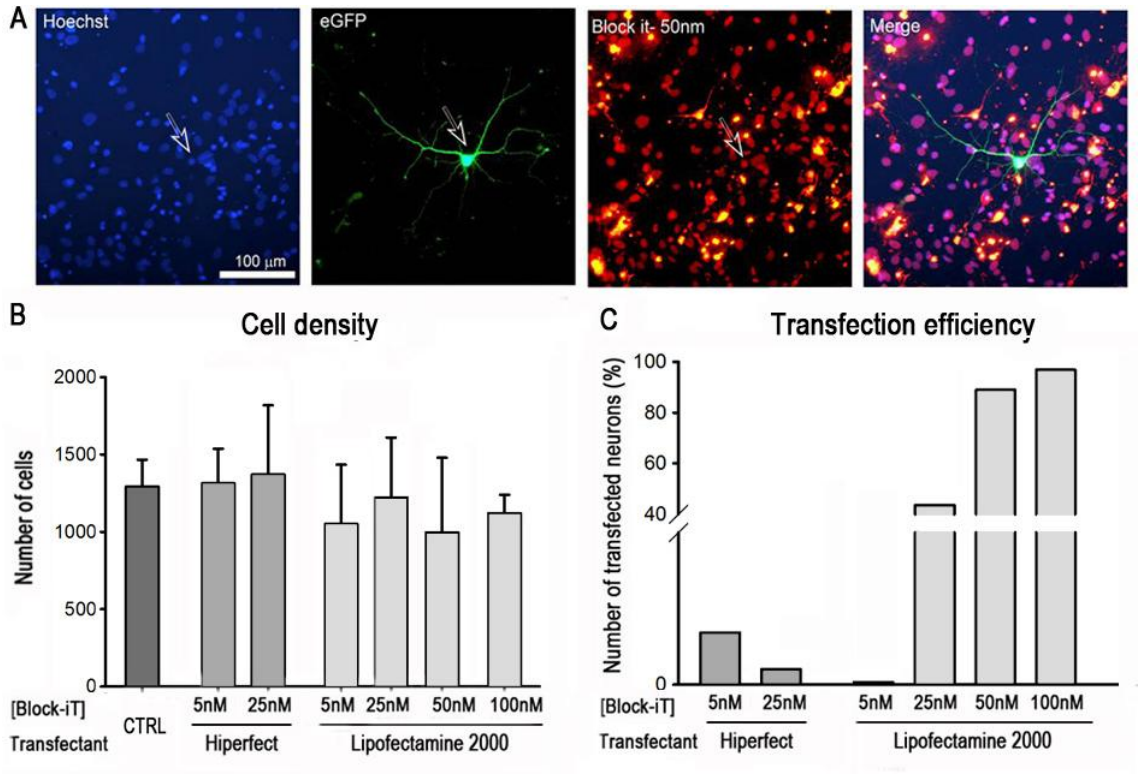


**Fig.10:** Optimization of hippocampal neuronal transfection with BLOCK-iTTM. **(A)** Example of a BLOCK-iT-positive neuron. A panel of a hippocampal neuron at 6 DIV co-transfected with pGFP and 50nM BLOCK-iT (nuclear red-stained) is presented using Lipofectamine transfection reagent and nuclear stained with Hoëchst. **(B)** Cell density, measured for each condition, shows no toxic effect of BLOCK-iT to any concentration. **(C)** Transfection efficiency obtained with transfection of rat hippocampal neurons with different concentrations of BLOCK-iT (5nM, 25nM, 50nM and 100nM) using either Lipofectamine or Hiperfect transfection reagents shows the elevated transfection efficiency for the highest concentrations of BLOCK-iT (50 and 100nM) when transfected with Lipofectamine 2000. Results represent quantification of two independent experiments and are expressed as percentage (mean ± St Dev).

Taking into account that the concentration of BLOCK-iT should be extrapolated to those for miRs mimics and inhibitors to posterior morphological measurements is crucial that such concentration achieve real high transfection efficiency, in order to be sure that our measurements represent reliable results. Since information provided by

supplier refers that concentration used to achieve an optimal activity for miRs inhibitors should be higher to those used for miRs mimics we decided to use miRs mimics at 50nM and inhibitors at 100nM.

***Evaluation of miRNA activity***

After choosing the appropriate oligonucleotides concentration to guarantee neuronal transfection was further evaluated their activity. Since miRNAs bind to mRNA target mediating mRNA degradation or inhibition of translation (Lang et al., 2012), their activity can be correlated with the expression level of the target protein. Thus, is essential the quantification of their expression after transfection with miRNAs mimics or inhibitors, comparing it with basal levels obtained from neurons control.

With this purpose, it was mandatory the search for validated mRNA targets of mir-124, mir-302b and mir-136. In addition to the miRNAs modulated by antidepressants, we have chosen two miRNAs previously described in literature as positive (mir-132) (Vo et al., 2005; Magill et al., 2010) and negative (mir-134) (Shratt et al., 2006) modulators of neuronal morphology. Search of miRNAs targets was performed with miRWalk database that comprises information of experimentally validated targets. This module had its last update on March 2011, crucial for reliable results. An extensive list of validated targets was provided by miRWalk database being chosen the target proteins involved in crucial pathways of neuronal development or connectivity (Table 6).

**Table 6: List of miRNAs and their validated gene targets provided by miRWalk Database.**

| miRNAs | Target | Biological processes | References |
|---|---|---|---|
| mir-302b | FMRP | mRNA transport and translation<br>Synaptic plasticity | Kao et al., 2010<br>Huber et al., 2002 |
| mir-214<br><br>mir-136 | PTEN | Adult neurogenesis<br>Synaptic plasticity | Amiri et al., 2012<br>Blair et al., 2012<br>Sperow et al., 2012<br>Yang et al., 2008. |
| mir-132 | MeCP2 | Transcription regulation | Su et al., 2012 |
| mir-134 | Limk1 | Regulator of actin dynamics<br>Synaptic stability | Foletta et al., 2004<br>Eaton et al., 2005 |

Another important aspect to take into account was the optimal time of incubation with miRNAs mimics/inhibitors in order to achieve the maximum activity of these oligonucleotides. Since previous works carrying out experiments with miRNAs with similar purposes to ours used 72h of incubation with miRNAs (Shratt et al., 2006) and information provided by supplier indicate it as a time in which miRNAs are stable and active, we have decided to do the experiments with 72h of incubation with miRs. In this manner, rat hippocampal neurons at 3 and 9 DIV were transfected with 50nM miRs mimics or 100nM miRs inhibitors using Lipofectamine2000 reagent and were fixed 72h after transfection using PFA 4%. In this way, it is possible to evaluate the miRNAs effect at 6 and 13 DIV, in young and mature neuronal stage (Dotti et al., 1988). After fixation, the coverslips were immuno-stained with the antibody against the corresponding protein target and also for cellular nuclei with Hoechst staining.

At this point, two kinds of measurements were made: one to evaluate the miRNA-targeted protein expression and another to evaluate neuronal survival. To measure the cell density, images of 5 fields of the coverslips were taken as described before and was done the count of the cell density of two different cultures treated either with miRs mimics or inhibitors. No significant changes were observed in the cell density between the control, cells treated with miRs mimics or with miRs inhibitors and cell density was between 130.000-150.000 cells/coverslip, what is important to show that the transfection treatment is not promoting cell death. In regard to the expression of the proteins targeted by miRNAs we have collected images with confocal microscope for neuronal densitometric analysis (see methods). Densitometric analysis allow us to determine the difference in the protein expression between control, miRs mimics and miRs inhibitors groups by quantification of protein fluorescence intensity. As the chosen miRNAs target proteins expressed at the soma was done the densitometric analysis on this specific area (see methods, Fig.9). Fluorescence intensity was measured through ROI Manager of ImageJ Software which analyzes the mean grey value of each stack of the chosen area. The mean grey value for each neuron was everytime normalized through the subtraction of the mean grey value of the background for each field, giving the true protein signal. Densitometric analysis showed a general decrease (between 17 and 36%) and increase (between 14 and 26%) on the protein expression when it was done the transfection with miRNAs mimics and inhibitors, respectively (Fig.11). Although there is not significant variances, such alterations on protein expression is reproducible for all miRNAs.

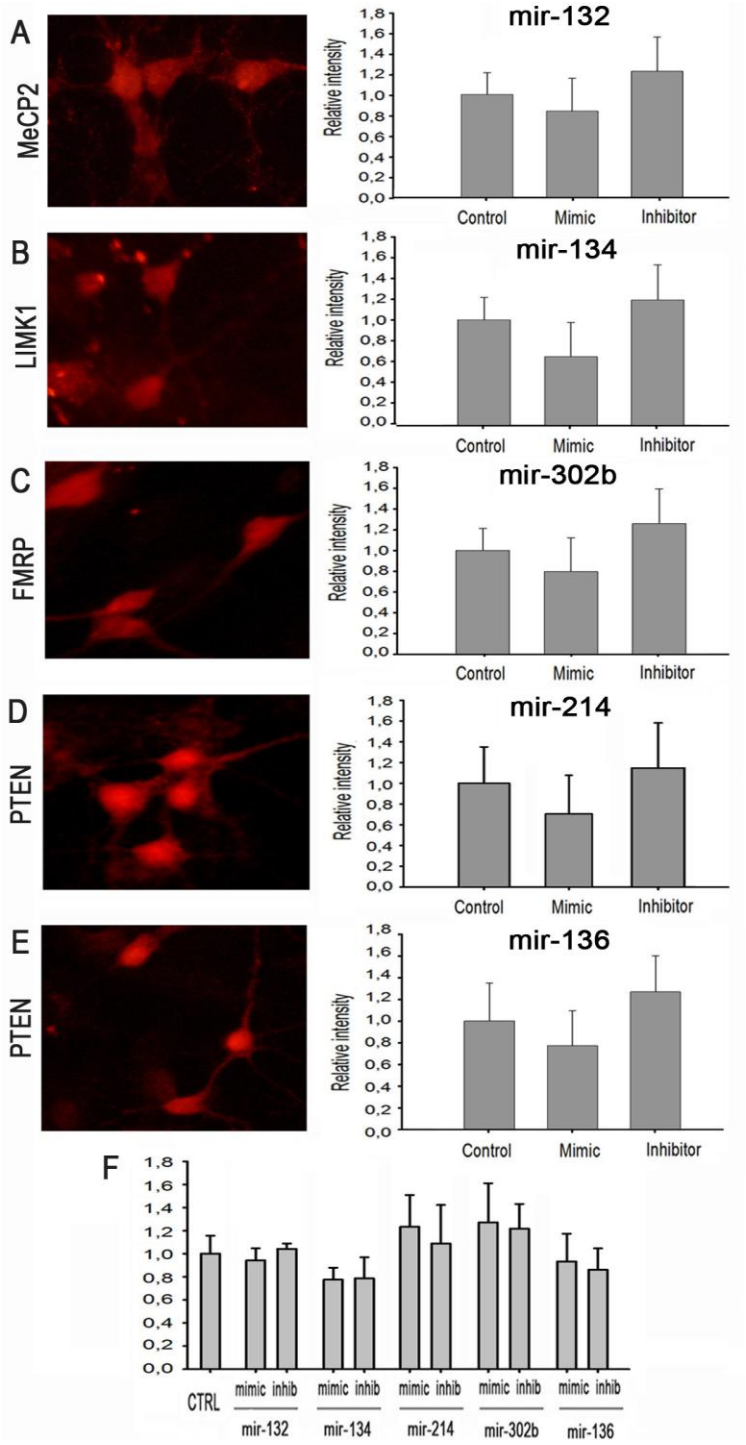


**Fig.11:** Protein expression quantification after transfection with miRNAs mimics and inhibitors. Protein expression levels quantified by densitometric analysis of the nuclear soma of hippocampal neurons incubated during 72h with mir-132 **(A)**, mir-134 **(B)**, mir-302b **(C)**, mir-214 **(D)** and mir-136 **(E)** shows a general increase and a decrease on the expression of the protein target when it is used miRNAs mimics and inhibitors, respectively. Data was collected from 25 neurons. **(F)** Cell density of hippocampal neuronal cultures did not suffer significant changes with the miRNAs transfection implying that there is not a toxic.

Finished the optimization of the neuronal transfection conditions with miRNAs oligonucleotides we have started the functional screening in vitro of the miRNAs effects on neuronal morphology, survival and BDNF translation.

### *Effect of miRNAs on neuronal morphology*

As miRNAs can target many genes involved in the establishment of neuronal morphology we had focus our attention on neuronal and spine morphology of hippocampal neurons treated with miRNAs mimics or inhibitors. Therefore, was measured the neuronal total dendritic length (µm) and the number of primary and secondary dendrites (see methods). These analyzes were performed through co-transfection of rat hippocampal neurons at 3 and 9 DIV with miRNAs mimics/inhibitors and GFP, in which miRNAs were incubated for 72h. At 6 and 12 DIV the coverslips were fixed with PFA 4% and images were collected with epifluorescence microscope and further analyzed with NeuronJ plugin of ImageJ software. The neuronal morphology was done in blind and in the most conservative manner possible. The primary dendrite was measured from its inception (the point of intersection between the primary dendrite and the soma) to its end, the farthest point of the dendrite. Secondary dendrites were measured in the same way, from its beginning (the point of intersect between the secondary and primary dendrites) and its end. To define the total dendritic length (µm) of each neuron was done the sum of the length of all dendrites. Finally, the total dendritic length for each miRNA mimic/inhibitor was presented as mean ± St Error. Statistical analysis with t-test and one way ANOVA were performed to verify if there were significant differences between miRNA treatments and control.

Neuronal morphology at 6 DIV did not change with the up or down-regulation of miRNAs, instead, at 12 DIV we can observe a statistically significant variance in the dendritic length of both mir-132 and -134. With miRs mimics was observed an increase on the total dendritic length for the mir-132 and a decrease for the mir-134. However, any significant changes were observed in the number of primary and secondary dendrites.

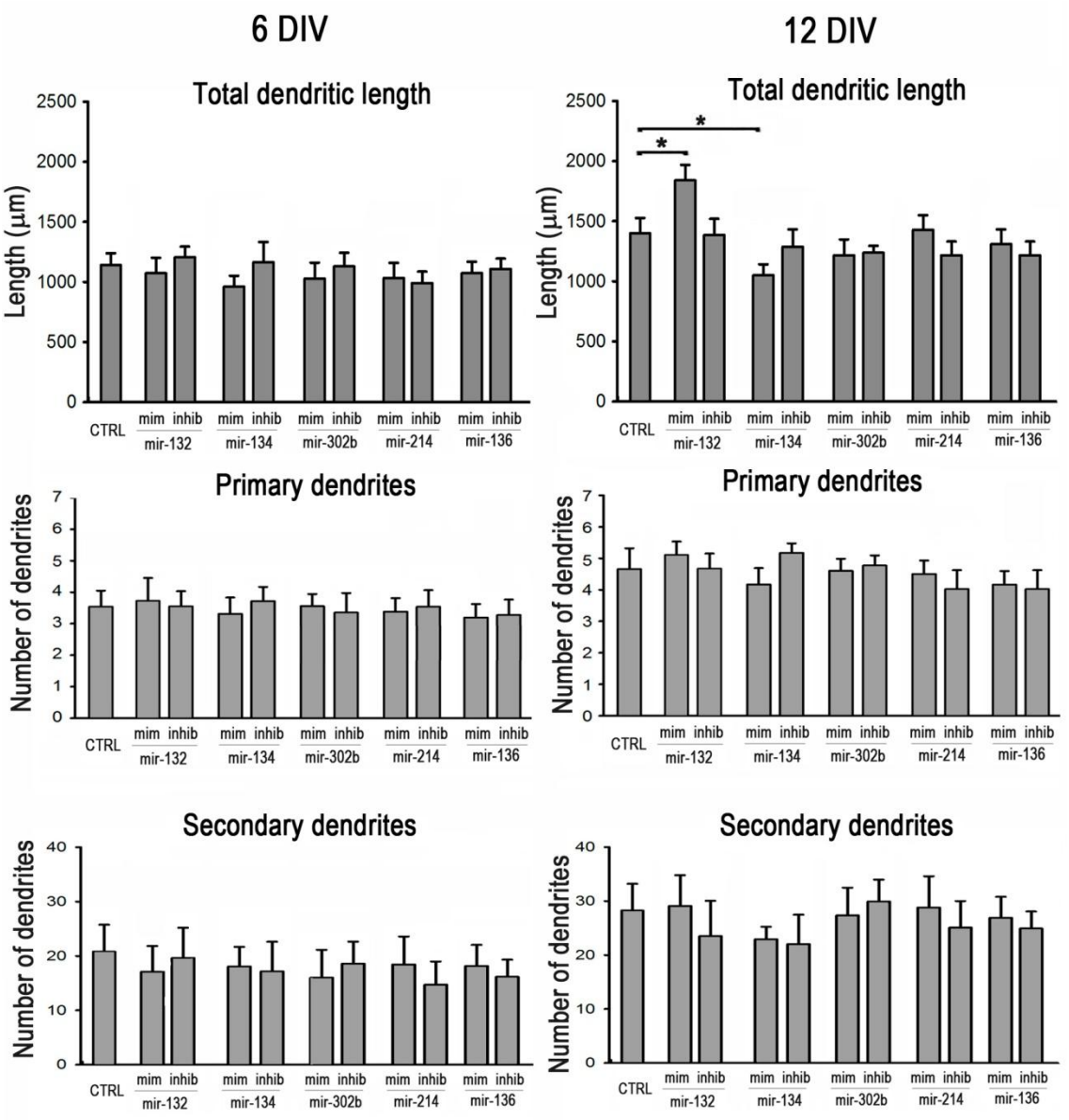


**Fig.12:** Effect of miRNAs expression in the morphology of hippocampal neuronal cultures. Total dendritic length (µm), primary and secondary dendrites are represented as mean ± St error. Quantification of total dendritic length and branches was made from at least 20 neurons. The statistical significance between mimics, inhibitors and controls neurons was evaluated using t-test where ***, P ≤0.001, **, P≤0.01, and, *, P≤0.05.

### *Effect of miRNAs in cellular viability*

Previous studies have demonstrated the role of miRNAs on cellular viability either by causing cell death (Fang et al., 2012) or cell proliferation (Zhong et al., 2012). In this way, we have tested the effects of the miRNAs on the viability of PC12 cells (pheochromocytoma cells), proliferative cells that allow us to quantify both cell death and cell proliferation ratios. PC12 cells grew in RPMI medium supplemented with 10% of horse serum and 5% of FBS in 25cm2 flasks and medium was replaced every 2-3 days in order to provide new nutrients to cells. Reaching a high cell density, PC12 cells were seeded into 96-well plate in RPMI medium low supplemented (5% HS and 2% FBS) which induces cell attachment at a cell density of $1 \times 10^6$. After one day, cells were transfected with 50nM of miRs mimics and 100nM of miRs inhibitors as previously described (see methods). Evaluation of miRNAs effect on cellular viability was carried out at two different times of incubation with the oligonucleotides, 48 and 72h. After incubation was performed the MTT assay, a colorimetric assay that permits to evaluate the activity of the reductase enzymes that convert MTT dye into formazan crystals in living cells (Tim Mosmann, 1983). Absorbance of the samples was measured using a plate reader at 560 and 600nm. Statistical analysis shows a significant decrease on the cellular viability at 48h of incubation with the miRNAs mimics/inhibitors of about 50% which demonstrates the cytotoxicity of the treatment for this cell line; however, the cellular viability shows an increase at 72h in comparison with the previous results and in some cases cellular viability is practically restored (Fig.13). Unfortunately, MTT assay doesn´t show any specific effect for the miRs mimics and inhibitors but instead a general cytotoxicity effect for the PC12 cells transfection with miRNAs oligonuceotides.

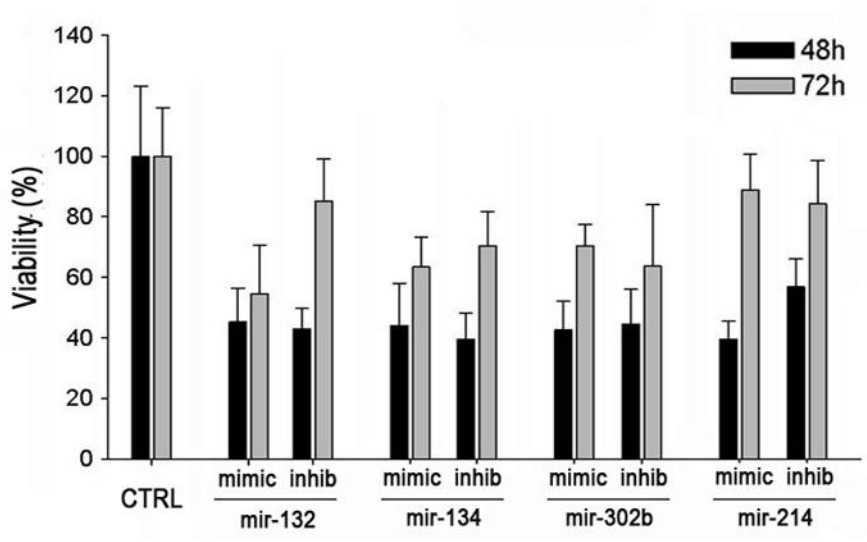


**Fig.13:** MTT assay performed to evaluate the effects of miRNAs on cellular viability. Two times of incubation with the miRNAs mimics and inhibitors were used (48 and 72h) and absorbance was read at 560 and 600nm. No specific effect on cellular viability is observed but rather the cytotoxicity of the treatment. Data were collected from 2 independent experiments and are represented as mean ± St Error.

### *Effect of miRNAs on BDNF translation*

Besides evaluation of miRNAs effects on neuronal morphology and cellular viability, we have even evaluated their effects on BDNF translation. Many miRNAs target BDNF inducing its expression decrease, which may explain the neuronal plasticity impairment observed in depressed patients (Blugeot et al., 2011). Therefore, is important to understand what kind of consequence the miRNAs modulated by antidepressant drugs have on BDNF mRNA. In this way, rat hippocampal neurons transfected at 3 and 9 DIV and incubated for 72h with miRs mimics/inhibitors were immuno-stained with antibody against BDNF (Sigma). After that, images collected with 60X objective of confocal microscope were analyzed with Imagej Software to do densitometric analysis to quantify fluorescence intensity of BDNF protein in control, miRs mimics and miRs inhibitors groups. Quantification of BDNF protein was done in

neuronal soma of hippocampal neurons as described at methods. In general, BDNF expression after incubation with miRNAs during 72h does not change significantly. Nevertheless, changes verified in the BDNF expression tends to follow a similar behavior both in soma and dendrites since it is observed that a decrease on the protein level in the soma as a correspondence decrease on the protein level on the dendrites. An important observation is that protein levels for miR-134, a modulator of the BDNF expression, demonstrate an alteration in its expression level, being verified an increase with the miRs mimics and a decrease with miRs inhibitors (Fig.13).

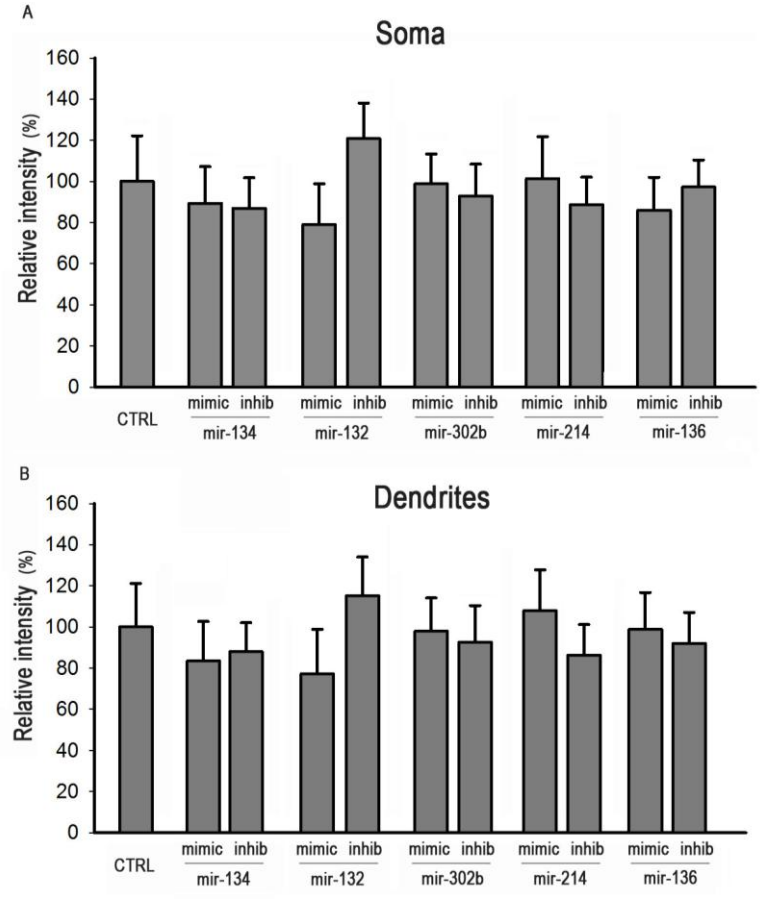


**Fig.14:** Effect of miRNAs on BDNF expression levels. Densitometric analysis represented as relative intensity of the protein expression from the soma **(A)** and dendrites **(B)** of hippocampal neurons transfected with miRNAs oligonucleotides does not show significant variances in the BDNF expression levels; however, for miR-134 it is observed a tendence for the increase and decrease of BDNF expression caused by miRNAs inhibitors and mimics, respectively.

***Bioinformatic analysis for miRNAs binding sites into BDNF gene***

MiRNAs can modulate the BDNF protein expression directly by targeting its gene or indirectly by targeting other genes involved in its transcription or translation mechanisms. The knowledge of miRNAs targets is essential to understand which mediators are involved in the mechanism of many diseases. As mentioned above, BDNF is decreased in depressed patients (Blugeot et al., 2011) being its expression level restored when it´s administrated antidepressant drugs (Ubhi et al., 2012), thus, it is important to know which miRNAs can target BDNF regulating its expression in the brain. Bioinformatic analysis emerges in this field as an important tool to search for miRNAs that have as target the BDNF mRNA. To obtain this information, the search was done through miRWalk database, which provided not only the miRNAs experimentally validated to target BDNF mRNA but also the miRNAs predicted to target it. Predicted targets are achieved through the study of the possible interactions between miRNAs and all known genes, being provided the interaction sites in the complete sequence of the gene (promoter, 5' UTR, CDS and 3' UTR). The possible miRNA-mRNA interactions are given by 9 different algorithms (miRWalk, Diana-microT, miRanda, miRDB, PICTAR, PITA, RNA22, RNAhybrid and Targetscan), which ensure a powerfull analysis. For each miRNA or gene target is provided a wide range of information is provided, such as: number of algorithms that recognize a specific complementary between miRNA-target, P-value of such match, number of nucleotides of miRNA seed sequence, position of the binding site, between others. From the extensive list of predicted miRNAs to target BDNF was done the selection of the miRs in agreement with the following parameters: recognition of the interaction miRNA-mRNA for at least 2 algorithms and miRNA seed sequence of at least 8

nucleotides. The binding sites of miRNAs were given for the BDNF transcript variant 1 of Human, the longest transcript of the BDNF gene.

Regarding validated BDNF-target miRNAs, the list of miRs was once again achieved by miRWalk database. Nevertheless, their precise binding sites were obtained by microRNA.org, a database that allowed us to obtain the binding site and its precise aligment between miRNA and target mRNA. The information collected by these miRNAs databases concerning the miRNAs targeting BDNF (either already validated or merely predicted) was summarized both in the figure 14 and the table 7. Among the validated and putative miRNAs to target BDNF gene is visible the higher affinity of miRNAs for the 3' UTR.

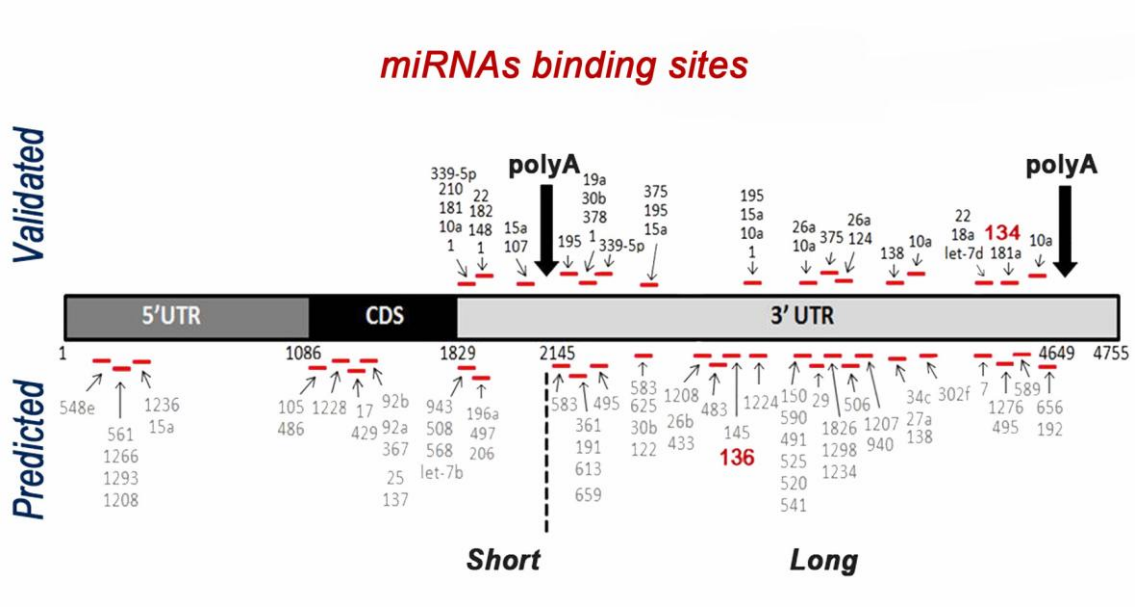


**Fig.15:** miRNAs binding sites into the Human BDNF transcript variant 1. Bioinformatic analysis for miRNAs binding sites shows a strong interaction between miRNA and BDNF mRNA in the 3´UTR. In the upper part is shown the binding sites for the experimentally validated miRNAs is shown while in the lower part are shown the miRNAs predicted to target BDNF. Repetition of microRNAs represents the multiple binding sites of the miRNAs. Notice that highlighted miRNAs are those evaluated in this study.

**Table 7:** Number of miRNAs targeting a specific region of BDNF gene.

| | 5'UTR | CDS | 3'UTR | |
|---|---|---|---|---|
| | | | Short | Long |
| Predicted | 7 | 10 | 7 | 39 |
| Validated | 0 | 0 | 10 | 18 |
| Total | 7 | 10 | 17 | 57 |

The preferential capacity of miRNAs to bind to the BDNF mRNA 3'UTR, especially its long portion, may have important consequences in the expression level of the two populations of BDNF transcripts either with the short or long 3'UTR. For this reason, it was important to understand the role of these two types of transcripts.

### 4.2. Role of 3'UTR long of BDNF mRNA in neurons

BDNF gene has a complex transcription mechanism, which can originate different isoforms of BDNF through alternative splicing (Aid et al., 2007). Likewise, alternative polyadenilation creates two populations of transcripts that carry either short or long 3'UTR. The structural and functional meaning on neuronal population of such transcripts is still a topic in debate. To determine their function, a mice strain specifically originated to lack the long 3'UTR- BDNFlox mice was created. The absence of the 3'UTR long has allowed us to evaluate its involvement in the neuronal and spine morphology and plasticity.

The neuronal morphology and plasticity was assessed through neuronal hippocampal neurons from $BDNF^{lox/lox}$ (referred as LOX), $BDNF^{lox/+}$ (referred as HET) and $BDNF^{+/+}$ (WT) mice, maintained in culture up to 6, 9 and 12 DIV. In this way it´s possible to study three different neuronal stages: young, adult and matured neurons (Baj, Patrizio and Tongiorgi, *in Submission*). Hippocampal neurons were transfected at 5, 8 and 10 DIV being fixated at 6, 9 and 12 DIV in PFA 4%. Subsequently, coverslips were immuno-stained with antibody against MAP2 and stained with Höechst. Images were collected in epifluorescence microscope and neuronal measurements were performed with NeuronJ plugin of the Imagej Software. For neuronal morphology was measured the length of the primary and secondary dendrites to achieve the total dendritic length; moreover, also the number of primary and secondary dendrites as well as branching points was count for each genotype and time point. Statistical analysis showed a significant reduction on the total dendritic length (μm) of the BDNFlox mice at 6 and 12 DIV in comparison to heterozygous or WT mice, that don´t have any difference between them. Nevertheless, the number of primary and secondary dendrites and also branching points do not suffer significant changes (Fig.14).

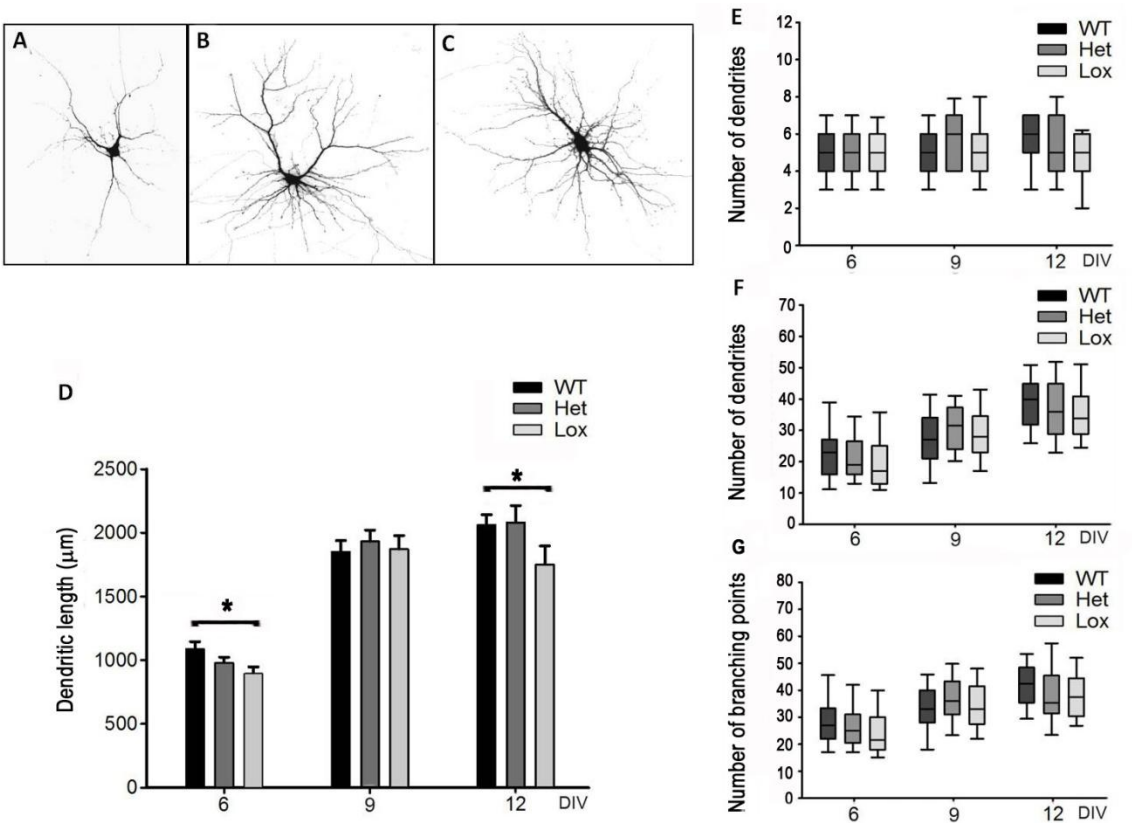


**Fig.16:** Hippocampal neurons of BDNFlox mice have a lower dendritic length but a normal number of branches. Representative images of hippocampal GFP-positive neurons at 6 **(A)**, 9 **(B)** and 12 **(C)** DIV. The images were taken through epifluorescence microscope using a 20X objective and analyzed with ImageJ Software to quantify neuronal morphology. Total dendritic length **(D)**, primary dendrites **(E)**, secondary dendrites **(F)** and branching points **(G)** of hippocampal neurons from $BDNF^{lox}$ (lox), $BDNF^{lox/+}$ (het) and $BDNF^{+/+}$ (wt) mice are represented as mean ± St error. Quantification of total dendritic length and branches was made from 44-56 neurons and at least 3 cultures for each time point and genotype. The statistical significance between the three genotypes was evaluated using t-test where ***, $P \leq 0.001$, **, $P \leq 0.01$, and, *, $P \leq 0.05$.

Measurements of neuronal and spine morphology were done in pyramidal hippocampal neurons, neurons characterized by their asymmetric dendritic arbor (Horton et al., 2006). For this reason, we have evaluated the polarization of the hippocampal neurons of each genotype in order to see if there is any change in the

polarization process. Neurons are considered as polarized when the apical dendrite has at least the double of the length of the other dendrites. Through this evaluation we have determined the percentage of polarized neurons and the contribution of each dendritic arbor to the total dendritic length using the Horton's method (Horton and Ehlers 2006) (see Methods). Any change is visible among the three genotypes, since the percentage of polarization and dendritic rank maintain in the same range of values for all of them.

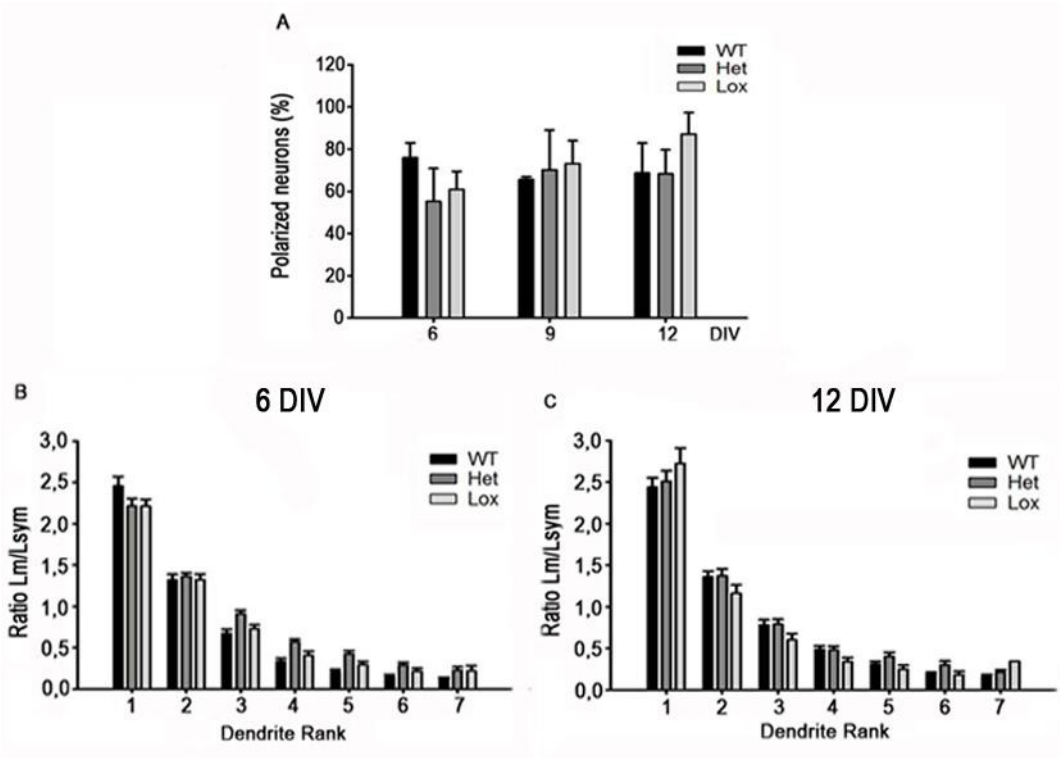


**Fig.17:** Polarity of hippocampal neurons in vitro. Percentage of polarized neurons (mean ± St error) do not shows significant differences between the three genotypes **(A)**. The contribution of each dendrite for the total dendritic length (expressed as ratio Lm/Lsym) at both 6 **(B)** and 12 **(C)** DIV is comparable among the three genotypes of mice. Dendrites are ranked longest to shortest. Data collected from 44 up to 65 neurons. The statistical significance among the three genotypes was evaluated using One Way ANOVA.

Afterwards, the spine morphology of each genotype was evaluated. Pictures of the spines were collected by GFP- positive neurons with 60X objective of confocal microscope. From each neuron we took two fields of 40µm in proximal and distal fields of the apical dendrite (see methods). Images were analyzed with ImageJ Software in order to achieve the number of stubby, thin and mushroom as well as the spine density (represented as number of spines per 10µm) present in each genotype. Concerning the spine density, in both genotypes was verified an increase on the spine number of both proximal and distal fields of the neurons as they grew up. $BDNF^{lox}$ mice tend to have a lower spine density, especially at later development stages. However, such difference is not significant. The qualitative analysis of the spine types which discriminate stubby, thin and mushroom spines observed in the apical dendrite points to morphological changes in the spines. There is a decrease in the stubby and an increase in the thin and mushroom spines number with the neuronal development. Among the three genotypes, changes in the proximal fields were observed; nevertheless, truncation of the long 3'UTR had a negative impact on the final stages of the spine maturation since $BDNF^{lox}$ mice have a decrease on the number of mushroom spines in the distal fields of matured neurons.

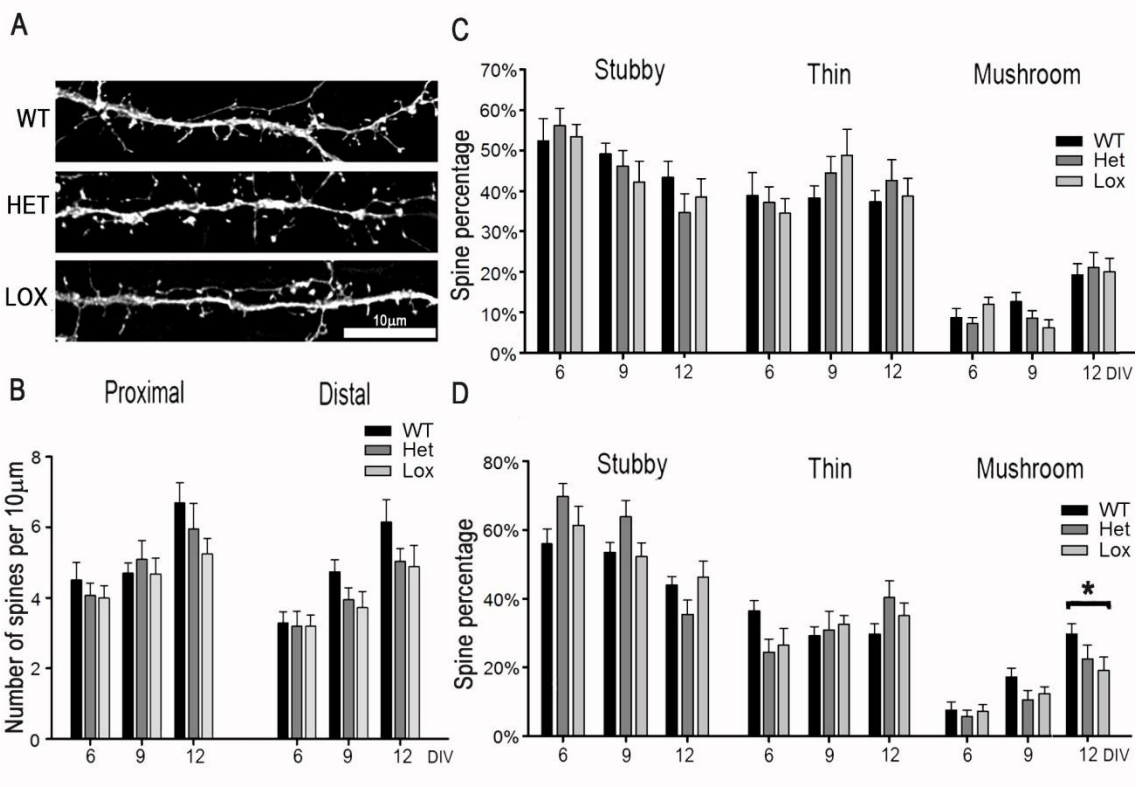


**Fig.18:** Spine maturation of hippocampal neurons from BDNFlox mice is impaired. **(A)** Representative confocal images of 40µm fields of distal fields of the apical dendrite of 12 DIV hippocampal neurons from $BDNF^{lox}$, $BDNF^{lox/+}$ (HET) and $BDNF^{+/+}$ (WT). **(B)** No significant changes were observed in the spine density, represented as the number of spines per 10µm, among the three genotypes. The percentage of spine types (stubby, thin and mushroom) in proximal fields **(C)** and distal fields **(D)** show a difference only in the percentage of mushrooms spines present in the distal fields of matured neurons. Measures are presented as mean + St Errors and statistical analysis were obtained using One Way ANOVA where ***, $P \leq 0.001$, **, $P \leq 0.01$, and, *, $P \leq 0.05$.

To evaluate the maturation of spines present in hippocampal neurons during neuronal development we have quantified the colocalization of Syn-I and PSD-95. With this purpose, cells were immuno-stained both against PDS-95 and SYN-1 and images were collected by confocal microscope. The quantification of the colocalization was performed following a threshold setting (see methods). Data were analyzed as the

percentage of PDS-95 signal that co-localizes together with Synapsin-1, regarding to the total output of PSD-95 signal. Following a statistical analysis using One Way ANOVA and all pairwise multiple comparison test, significant differences were observed between $BDNF^{lox}$ and WT mice at the three time points. The results show a decrease of approximately 40% in the colocalization PSD-95/SynI in hippocampal neurons of $BDNF^{lox}$ mice comparing with WT mice (Fig.19).

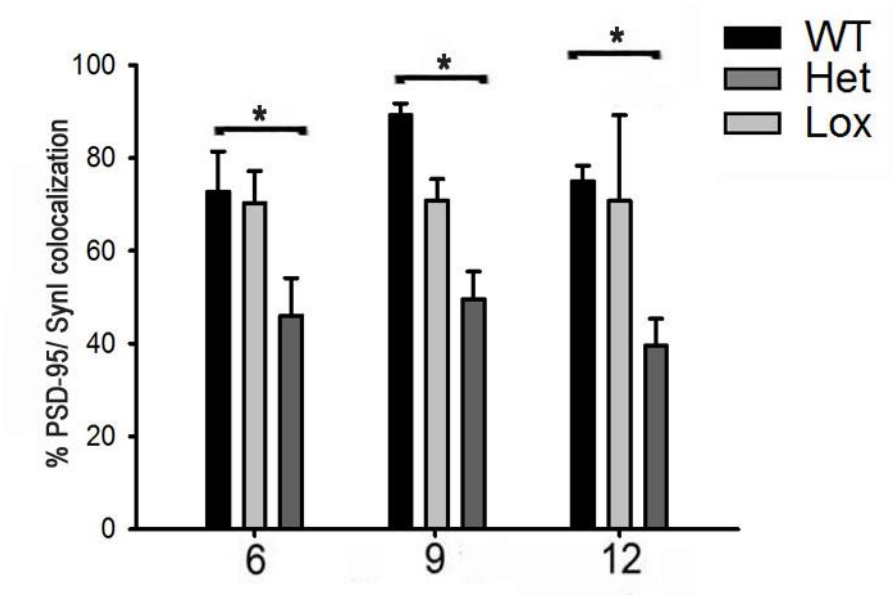


**Fig.19:** Colocalization of PSD-95 and Syn I. Quantification of the degree of the colocalization (represented as the percentage of colocalized PSD-95 with respect to the total PSD-95) shows significant variance between the BDNFlox and WT mice for all time points. Statistical analysis was done with One Way ANOVA followed by an all pairwise multiple comparison procedure with Holm-Sidak's method where ***, $P \leq 0.001$, **, $P \leq 0.01$, and, *, $P \leq 0.05$.

Depression is a mood disorder that affects proximately 121 milion people worldwide and represents one of the most common disorders to disability (World Health Organization). Many factors are involved in the onset of this disorder including dysregulation of miRNAs signaling (Saus et al., 2010). Since miRNAs can target and inhibit the expression of many genes, regulating a large spectrum of biologic processes, defects on their activity may have undesirable effects on brain function. Diverse types of antidepressants can in turn modulate miRNAs expression. Experiments performed by our collaborators at the University of Milan have shown that antidepressant drugs administrated in adult rats for two weeks modulate the expression of several miRNAs, either by promoting their increase or decrease. Thus, the functional meaning of the alterations in the miRNAs expression pattern caused by antidepressant drugs emerges as an important field of research.

In order to understand the role of miRNAs modulated by antidepressants in the neuronal cells and, consequently, in brain function, three miRNAs were selected from the list of the miRNAs compiled by microarrays analysis carried out on brain RNA extracted from adult rats treated with the drugs. The selection of these miRNAs was done taking into account the following parameters:

-High fold change of its expression levels following the antidepressant treatment;

-Have a validated gene target for the evaluation of their activity.

The selected miRNAs mir-136, mir-302b and mir-214 were further evaluated for their effects on neuronal morphology and survival as well as BDNF translation in vitro.

Evaluation of miRNAs effects was performed using rat hippocampal neuronal cultures, which were subject of transfection with miRNAs mimics and inhibitors. These are synthetic oligonucleotides which mimic endogenous mature miRNAs: the mimics produce a decrease of gene expression while the inhibitor lead to an inhibition of the endogenous miRNA, allowing us to achieve conditions similar to the endogenous up- or down-regulation of miRNAs to further gene expression and phenotypic analysis. In the absence of an established protocol to neuronal transfection with miRNAs, it was mandatorily to set-up the conditions to do these experiments.

To the establishment of the protocol we have followed a specific line of reasoning to guarantee not only the neuronal transfection with the miRNAs but also their inhibitory activity into the cells. According to this, we started to identify the best concentration of miRNAs to transfect the largest number of neurons in culture. Since these oligonucleotides are not fluorescently labeled and therefore it is impossible to track them, it was necessary the use of the BLOCK-iT (Invitrogen), a red-labeled dsRNA oligomer with similar length, charge and configuration to the miRs, without a homology to any known gene avoiding the silencing of neuronal proteins. We experimentally verified that the correct concentration of miRNAs oligos to reach the highest neuronal transfection efficiency is either 50 or 100nM. Moreover, evaluation of the impact of the used concentrations of BLOCK-iT on neuronal survival did not show any significant effect on cell density. However, transfection with Lipofectamine2000 reagent showed a slight toxic effect on neurons, since cell density in these cases is decreased, but not significantly. These results show that such concentrations of synthetic oligonucleotides are not letal for neuronal cells.

Since information provided by the supplier refers that concentration of miRNAs inhibitors should be higher than the concentration of miRNAs mimics to obtain the

inhibition of the endogenous miRNA, we had to choose the concentration of 100nM for miRNAs inhibitors and 50nM for mimics.

As miRNAs are small RNA molecules with affinity to mRNAs promoting their degradation or translation inhibition (Lang et al., 2012) and consequent decrease on protein expression, we focused our attention in the quantification of the target proteins expression. With this purpose we searched for experimentally validated targets for the three miRNAs to be evaluated. Through bioinformatic analysis using miRWalk database we have chosen one target for each miRNA (see results, table 5) based on its neuronal expression and involvement in crucial pathways of neuronal development and connectivity. After neuronal transfection with both miRs mimics and inhibitors we have quantified the protein expression by densitometric analysis. This approach allowed us to quantify the expression levels of the protein target by miRNAs and correlate it with miRNAs activity. As we achieved a decrease of 17-36 % and an increase of about 14-26 % upon miRs mimics or inhibitors transfection, respectively, we can assume that miRNAs have activity in neurons. These small changes are similar to those mediated by endogenous miRNAs and are in agreement with the characteristic of miRNAs to control biological processes through slight variations in the levels of target proteins. Furthermore, as miRNAs have many possible targets, miRs mimics/inhibitors may affect other mRNAs having a general action on the total endogenous mRNAs targets, explaining why we do not see strong changes in the selected protein target. In these experiments we have also evaluated the effect of miRNAs transfection on neuronal survival but we did not observe any significant change. This result is important to demonstrate that transfection with miRNAs does not have a cytotoxic effect and that protein decrease produced by miRNAs is not being prejudicial to neuronal survival.

The importance of miRNAs on the modulation of the neuronal morphology is generally accepted (Shratt et al., 2006; Magill et al., 2010; Agostini et al., 2011; Zhang et al., 2011; White et al., 2012). As antidepressant drugs altered the expression of some miRNAs it was hypothesized that their therapeutic effects are achieved, at least in part, by miRNAs-mediated gene regulation benefiting depression´s recovery. Morphological measurements of hippocampal neurons at 6 and 12 DIV have shown a modulation effect for the mimics-132 and -134. These miRNAs were previously described in literature as positive (mir-132) (Vo et al., 2005; Magill et al., 2010) and negative (mir-134) (Schratt et al., 2006) modulators of neuronal morphology, since they can increase or decrease, respectively the neuronal arborization outgrowth. Taking this into account, our results in which the mimic-132 and -134 produce an increase and decrease of the total dendritic length, respectively, is in agreement with previous studies. However, inhibitors did not show any impact on the neuronal morphology. Our data on miR-inhibitors can be compared only with a previous study in which mir-132 was genetically ablated by gene mutagenesis (Vo et al., 2005) showing significant impact on dendrites length. The divergent results may be explained by different technical bases of these studies and/or by a too low concentration of the miR-inhibitors used here.

Previous studies had present evidences for the role of miRNAs on cellular viability either by enhancing cell proliferation (Zhong et al., 2012) or causing cell death (Fang et al., 2012) through gene expression regulation. The evaluation of viability of PC12 cells (pheochomocytoma cells) at 48 and 72h demonstrated the cytotoxic effect of the miRNAs transfection for this cell line. In fact, at 48h was observed the decrease of about half of the cell survival; nevertheless, there was a recovery of cell survival at 72h which in some cases was similar to the control. The rescue of cell viability at 72h can be

explained by the higher capacity of proliferation of these cells after a first cytotoxic effect occurring upon the initial transfection.

Many miRNAs can target BDNF inducing its decrease, which may explain the neuronal plasticity impairment observed in depressed patients (Blugeot et al., 2011). Therefore, it is important to understand what impact the miRNAs modulated by antidepressant drugs may have on BDNF mRNA. The quantification of BDNF protein after incubation with miRs mimics/inhibitors demonstrate that, except for mir-132, for which is observed a tendency to modulate the BDNF expression (it is slightly increased with the mir-132 inhibitor and slightly decreased with the mir-132mimic), there are no changes in the BDNF expression for the others miRNAs. Results obtained with preliminary densitometric quantification on soma or full dendrites with miRNAs treatments did not show a strong variance in the BDNF expression. However, the miR-132 and mir-134 were previously described as modulators of BDNF expression (Shratt et al., 2006; Kawashima et al., 2010) and since miRNAs inhibitory action may modulate protein translation in different ratios among the neuronal cells in culture, evaluation of this parameter with another kind of approach or statistical analysis would be of great importance. For that reason, a new analysis of stained coltures is currently in progress and will give new insight at specific subcellular domains (Magill et al. 2010) involved in BDNF translation miRNA regulated.

The modulation of the neuronal morphology by miRNAs raises the question of the involvement of BDNF on it, since it is recognized the implication of this neurotrophin in morphological features. Thus, it is important to know whether morphological alterations mediated by miRNAs are due to BDNF expression regulation. A significant number of miRNAs target BDNF gene and an even higher number of these molecules are predicted to target it. Through bioinformatics analysis it

was possible to get the miRNAs that are already validated or only predicted to target BDNF gene and regulate its expression. Generally, such regulation is mediated by the interaction miRNA-mRNA that is done mostly in the 3'UTR. In fact, among the miRNAs validated or merely predicted to target BDNF, 65 target the 3'UTR and the majority of them in the long portion of the 3'UTR region. This observation may imply a differential targeting and regulation of the two BDNF transcripts populations having either a short or a long 3'UTR. Therefore, translational inhibition of BDNF proteins originated by the two types of transcripts may have different implications in the development or homeostasis of neuronal cells.

While initially, a previous studies have presented evidence for the differential target of the BDNF transcripts according to the 3' UTR in resting neurons (An et al., 2008), two recent study showed that both short and long 3'UTR BDNF transcripts can be targeted to dendrites (Baj et al., 2011; Yoneda et al., 2011). Dendritic targeting is apparently due to cytoplasmic polyadenilation element (CPE)-like sequences present in both the short and the long 3' UTRs (Yoneda et al., 2011); however, it was observed that only transcripts with the long 3'UTR can reach the distal part of neuronal cells (Baj et al., 2011).

Experiments were performed in hippocampal neurons from $BDNF^{lox}$ mice (Gorski et al., 2003) in which the BDNF gene is truncated for the long 3'UTR and, thus, merely expresses the short 3´UTR BDNF transcripts, allowing us to evaluate the role of the BDNF proteins translated from the two populations of BDNF mRNA transcripts. Truncation of the long 3'UTR of BDNF resulted in a smaller dendritic length of neuronal cells in either young or already matured neurons, i.e. at 6 and 12 DIV. However, it is important to notice that there were no differences between WT, heterozygous and $BDNF^{lox}$ mice at 9 DIV. Nevertheless, the number of primary and

secondary dendrites and branching points did not change in the absence of the long 3' UTR at any time point. Taken together, these observations indicate that long 3' UTR is necessary for the growth of the final regions of the dendrites and that such role is more important at dynamic stages of neuronal development.

Another parameter analyzed in our study was the polarization of neuronal cells in culture. Pyramidal hippocampal neurons are characterized by their asymmetric dendritic arbor (Horton et al., 2006). The dendritic dynamic is represented by the extension and retraction of the dendrites and it is during this developmental stage that one dendrite becomes larger and bigger than the surrounding dendrites. The evaluation of this parameter demonstrat that truncation of long 3'UTR does not have implications on neuronal polarization since the percentage of polarized neurons as well as the dendrites rank, which evaluate the contribution of each dendrite to the final dendritic length, were similar among the three genotypes. Afterwards, spine morphology of WT, heterozygous and $BDNF^{lox}$ mice was evaluated in order to see if the long 3'UTR has an important role in the structure and function of dendritic spines. The categorization of the spines present in the proximal and distal fields of the apical dendrite demonstrated that there is a switch of spine types during neuronal development. At earlier stages, stubby spines are present in a higher number and then they diminish with maturation giving rise to thin and mushroom spines, what is in agreement with the process of spine development (Papa et al. 1995). Although this development was observed in all genotypes, we found at late developmental stages (12 DIV) a lower number of mushroom spines in distal fields of $BDNF^{lox}$ mice hippocampal neurons. Concerning the spine density at both proximal and distal dendritic fields, we did not observe any changes in the absence of the long 3´UTR.

Besides a qualitative and quantitative analysis of the spine types we also quantified the colocalization between PSD-95 and SynI, to evaluate the presence of synapses. This evaluation shows a decrease of about 25% and 35% in the colocalization in $BDNF^{lox}$ mice at 6 and 12 DIV, respectively, which means that truncation of long 3'UTR has severe consequences in the connectivity of hippocampal neurons.

Taken together, these results suggest that not only the spine maturation in terms of the development of spines to mushroom spines, the most mature dendritic spines, but also the neuronal connectivity is dramatically impaired. However, it is important to notice that negative changes in spine function takes place only in distal fields, which goes in agreement with the target of the BDNF proteins translated from the long 3'UTR mRNA to the dendrites and its redundancy with the 3'UTR short in shaping the spine morphology at proximal fields. As the short 3'UTR BDNF cannot reach the most distal parts of the dendrites, it is in that area that the absence of the long 3'UTR is visible.

A very recent study performed by Kaneko et al. (2012) has presented evidences in agreement with our results concerning the impairment of spine maturation. However, a divergent but interesting observation regarding dendritic spines morphology was in what concerns the impact of long 3'UTR in the spine density. In this study, cortical neurons only with short 3'UTR BDNF transcripts presented an increase in the spine density in the distal fields (Kaneko et al., 2012) which is explained by the authors as a result of the deficient spine pruning in this situation. Dendritic spines are overproduced at postnatal life especially in cortical neurons, but at a certain point there is an elimination of up to 40% of the spines while the remaining spines become mature and support the synaptic activity in the brain (Marin-Padilla, 1967; Huttenlocher, 1979). Thus, it is suggested that the lack of the long 3' UTR may prevent the spine pruning (Kaneko et al., 2012). In this manner, comparing with neuronal cells with the two

populations of BDNF transcripts where there is a normal spine pruning, the spine density of $BDNF^{lox}$ mice is higher since there is not an elimination of the spines. Although there is an established role for spine pruning in the cortex, there is a remarkable lack of information regarding spine pruning in hippocampus. In fact, although without significant variances, a slightly decrease in the $BDNF^{lox}$ mice was observed in our study. This result can be due to a different spine development in the hippocampus. A previous study carried out in our lab in which we evaluated the spine development, showed a growing number of spines during 15 days in vitro, at any time point during development was observed spine pruning and consequent spine decrease (Baj, Patrizio, Montalbano, Sciancalepore and Tongiorgi, in preparation). So far, spine pruning in hippocampal neurons are only connected to synaptic plasticity after a procedure of extinction (Garin-Aguilar et al., 2012) but not in a normal spine development.

As both populations of BDNF transcripts are transcribed in the same manner, only differing in the polyadenilation processing (Aid et al., 2007), truncation of the long 3'UTR does not affect the transcription of the BDNF gene but rather the targeting of dendritic distal parts. However, it remains unclear how this long 3'UTR affect both translation and active mRNA transport in dendrites.

The results obtained in this study have highlighted important aspects concerning the role of the 3'UTR long in the function and development of hippocampal neurons. $BDNF^{lox}$ mice, truncated for the long 3' UTR BDNF, showed deficiencies in total dendritic length of hippocampal neurons and even structural and functional synaptic-modifications. These results are important to many fields of research, including the study of the depression´s mechanism. Since miRNAs are dysregulated in depressed patients, it was important to evaluate their effects on neuronal cells. Our results suggest that miRNAs modulated by antidepressant treatment during two weeks do not have any effects on neuronal morphology and survival. Their effects on BDNF expression levels are still unclear and new statistical analysis should be performed, however, it cannot be discarded the possibility of a BDNF expression modulation, which could impact mostly at synapses. Since many miRNAs are putative or validated targets of BDNF gene, mainly in the 3´UTR, it can be possible that miRNAs upregulated in depression could target the long 3'UTR of BDNF explaining, together with the dysregulation of other proteins, the diminished synaptic plasticity observed in depressed patients.

As evaluation of the miRNAs effects on the modulation of the BDNF translation in hippocampal neurons through densitometric analysis did not present conclusive results, it is already ongoing another type of analysis based on a different statistical approach. Moreover, the quantification of BDNF levels will be furthermore explored through Western Blot or ELISA techniques in order to confirm the results from densitometric analysis. Likewise, the number of hippocampal neurons for the assessment of the proteins expression levels will be increased to achieve a more representative ratio for the protein level after up- or down- regulation of miRNAs.

Finally, a new set of experiments using a multiple miRNAs modulation approach to study in vitro a condition of RNA regulation will be done to achieve a more representative depression status.

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