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miRNA in the regulation of synaptic function

Dissertação de Mestrado em Biologia Celular e Molecular, orientada pela Professora Doutora Ana Luísa Carvalho e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Universidade de Coimbra

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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 da Professora Doutora Ana Luísa Carvalho (Departamento de Ciências da Vida, Universidade de Coimbra) e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

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Nothing in life that is great is easy. If it was easy then it wouldn't be great.

From *Who says you can't? You do* – Daniel Chidiac

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Abbreviations

4-AP	4-aminopyridine
5-HT	5-hydroxytryptamine
AGO	Argonaute protein
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid receptor
AP2	Adaptor protein 2
AP-V	(2R)-amino- 5-phosphonovaleric acid
Arc	Activity-regulated cytoskeletal associated protein
BACE1	APP-cleaving enzyme 1
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaMKK	CaM kinase kinase
CaMKKIIγ	Calcium/calmodulin dependent protein kinase IIγ
CLAP	Chymostatin, leupeptin, antipain and pepstatin
cLTP	Chemical-LTP
CREB	cAMP response element-binding protein
DHPG	R,S-dihydroxyphenylglycine
DIV	Days in vitro
DTT	Dithiothreitol
ECF	Chemifluorescence substrate
ECS	Extracellular solution
EGF	Epidermal growth factor
E-LTP	Early-LTP
Eps15	EGF receptor pathway substrate 15

ER	Endoplasmic reticulum
ERK	Extracellular signal–regulated kinases
FMRP	fragile X mental retardation protein
GABAA	Gamma-aminobutyric acid A
Gly	Glycine
GRIP1/2	Glutamate receptor-interacting protein 1/2
Gyki	2,3-benzodiazepine
HBSS	Hank's balanced salt solution
HFS	High-frequency stimulation
HS	Horse serum
KD	Knockdown
KO	Knockout
Limk1	Lim-domain-containing protein kinase 1
L-LTP	Late-LTP
LTD	Long-term depression
LTP	Long term potentiation
MAP1b	Microtubule associated protein 1b
MCPIP1	Monocyte chemotactic protein-1-induced protein-1
MeCP2	Methyl CpG-binding protein2
Mef-2	Myocyte enhancer factor-2
mEPSC	Miniature excitatory postsynaptic current
mGluR	Metabotropic glutamate receptor
miRNA	MicroRNA
MK801	Dizocilpine
mTOR	Mammalian target of rapamycin
MYOD1	Myoblast determination protein 1
NBM	Neurobasal medium

NMDAR	N-methyl-D-aspartate receptor
NPR	Neuronal pentraxin receptor
NSCTN	Nicastrin
NSF	N-ethylamide-sensitive fusion protein
PD	Postnatal day
Pen-Strep	Penicillin-streptomycin
PFC	Prefrontal cortex
PI3K	Phosphoinositide 3-kinase
PICK1	Protein Interacting with C Kinase 1
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl
PNPT1	Polyribonucleotide nucleotidyltransferase 1
Pol II	RNA polymerase II
PP1	Protein phophatase 1
Pri-miRNA	Primary miRNA
PSD	Postsynaptic density
PTEN	Phosphatase and tensin homolog
PUM2	Pumilio-2
PVDF	Polyvinylidene difluoride
RBPs	RNA-binding proteins
RISC	RNA-induced silencing complex
RNase III	Nuclear ribonuclease III
SDN1	Small RNA degrading nuclease 1
SENP1	Sentrin-specific protease 1
SERCA2	Sarco/endoplasmic reticulum calcium ATPase
siRNA	Small interfering RNA
SNARE	Soluble NSF attachment receptor

snoRNAs	Small nucleolar RNAs
STEP	Striatal-enriched tyrosine phosphatase
TACE	Tumor necrosis factor-a-converting enzyme
TARPs	Transmembrane AMPAR regulatory proteins
TBS	Tris-buffered saline
TBS-T	0.1% Tween 20 in TBS
TGS	Trisglycine-SDS
Tmod2	Tropomodulin 2
TNFα	Tumor necrosis factor α
TRBP	TAR RNA-binding protein
TrkB	Tropomyosin receptor kinase B
TTX	Tetrodotoxin
UTR	Untranslated region
VGluT1	Vesicular glutamate transporter 1
XRN	Exoribonucleases

Abstract

miRNAs are a class of small non-coding RNAs with about 22 nucleotides, that play a critical role in posttranscriptional regulation of gene expression, through mRNA degradation or translation repression. Generally, miRNAs recognize the 3' UTR of the target mRNAs by a partial and imperfect base-pairing except on the seed region, where a nearly perfect pairing occurs. These features provide to each miRNA pairing specificity and the capacity to regulate many mRNAs, allowing the establishment of a vast regulatory RNA network with flexible control of mRNA expression. Thus, it is not surprising that the miRNA system regulates processes such as neuronal development and synaptic plasticity. In fact, several miRNAs have been shown to locally regulate dendritic arborisation, spine growth and remodelling by targeting cytoskeleton regulators; and regulate also synaptic strength by directly targeting AMPA receptors or by targeting proteins involved in their trafficking. At synapses, changes in neuronal activity induce alterations on miRNAs levels, suggesting that neuronal-activity dependent regulation of miRNAs fine-tunes translation in response to synaptic plasticity, which underlies learning and memory formation. Dysregulation of this mechanism may ultimately contribute to the pathogenesis of cognitive impairments and neurodevelopment disorders. Therefore, it is important to clarify the functional role of miRNAs in different paradigms of neuronal activity.

Previous results from our group show that neuronal activity blockade in hippocampal neurons rapidly downregulates both miRNA-132 and miRNA-186 levels, suggesting that these miRNAs may participate in activity-regulated processes. Considering this indication, further studies were conducted about the expression pattern of these miRNAs in the hippocampus throughout development, how they are regulated by different paradigms of activity and what their impact is on synaptic function.

During neurodevelopment, both miRNA-132 and miRNA-186 have an increase on their expression levels. Conversely to miRNA-132, whose levels increase gradually but steadily in the hippocampus during development, miRNA-186 expression is stable in the first four postnatal weeks, and it expression increases steeply thereafter. Neuronal depolarization and cLTP regulate miRNA-132 expression levels, promoting an increase on its levels in cultured hippocampal neurons. On the other hand, mGluR-dependent LTD results in a rapid decrease on the expression levels of miRNA-186. Inhibition of miRNA-186 produced no effects on the amplitude or frequency of miniature excitatory postsynaptic currents in cultured hippocampal neurons, indicating that under basal conditions miRNA-186 does not affect synaptic function, but suggesting that this miRNA-186 may be relevant during synaptic plasticity. These results corroborate the idea that during synaptic plasticity different

subsets of miRNAs can be regulated differently, thereby fine-tuning the neuronal response to activity.

Resumo

Os miRNAs são uma classe de pequenos RNA não codificantes com cerca de 22 nuleótidos, cruciais na regulação pós-transciptional da expressão de genes, tanto através da degradação do mRNA como da repressão da síntese proteica. Os miRNAs reconhecem geralmente locais na 3'UTR dos mRNAs-alvo, onde se ligam de forma parcial excepto na *seed region*, onde a ligação é quase perfeita. Desta forma, a ligação mRNA-miRNA, para além de conferir especificidade, possibilita também que apenas um miRNA possa regular vários mRNAs, permitindo a criação de uma vasta rede de regulação do RNA com um controlo flexível da expressão de mRNA. Por esta razão, não é surpreendente que o sistema estabelecido pelos miRNA regule processos como o desenvolvimento neuronal e a plasticidade sináptica. De facto, vários miRNAs têm sido descritos como reguladores: da arborização dendrítica, do crescimento e da plasticidade de espículas, ao modularem a expressão de reguladores do citoesqueleto. Por outro lado, os miRNA podem regular a função sináptica, podendo ter como alvos diretos os recetores AMPA ou regulando os mesmos indiretamente, através de modelação de proteínas envolvidas no tráfego destes receptores. De notar, modificações na atividade neuronal podem promover o ajustamento nos níveis de miRNAs, sugerindo que uma regulação induzida por atividade promove o controlo preciso da síntese proteica em resposta à plasticidade sináptica, a qual está na base da aprendizagem e formação de memórias. Como consequência, a desregulação deste mecanismo pode contribuir para o aparecimento de disfunções cognitivas e de distúrbios psiquiátricos, sendo por isso importante clarificar as funções dos miRNAs nos diferentes paradigmas de atividade.

Resultados preliminares do nosso grupo mostram que bloqueio na atividade neuronal, em neurónios do hipocampo, rapidamente promove a diminuição nos níveis de ambos os miRNA-132 e miRNA-186, sugerindo que estes poderão participar em processos regulados por atividade. Tendo por base estes factos, foram realizados estudos complementares para determinar a expressão destes miRNAs, no hipocampo, ao longo do desenvolvimento, como são regulados por diferentes paradigmas de atividade e, consequentemente, os seus impactos na função sináptica.

De facto, durante o desenvolvimento neuronal, a expressão de ambos os miRNA-132 e miRNA-186 aumenta. Contrariamente ao miRNA-132, cujos níveis aumentam gradualmente mas de forma constante, no hipocampo, durante todo o desenvolvimento, a expressão do miRNA-186 é estável nas primeiras quarto semanas pós-natais, aumentado acentuadamente posteriormente. Despolarização neuronal bem com o cLTP regulam positivamente os níveis do miRNA-132, aumentando a sua expressão em neurónios do hipocampo. Pelo contrário, LTD dependente de mGLuRs resulta numa rápida diminuição nos níveis de expressão do miRNA-186. Não obstante, a

inibição do miRNA-186 não promoveu quaisquer alterações na amplitude ou frequência de correntes miniatura excitatórias pós-sinápticas em neurónio do hipocampo, indicando que em condições basais, o miRNA-186 não afeta a função sináptica, mas no entanto sugerindo que este miRNA poderá ser relevante durante a plasticidade sináptica. Desta forma, os resultados corroboram a ideia de que durante a plasticidade sináptica diversos conjuntos de miRNAs podem ser regulados diferentemente, e portanto, uma regulação precisa à resposta neuronal à atividade.

Introduction

Glutamatergic synapses and AMPA receptors

All our behaviours, thoughts, emotions, and memories are triggered by a hundred billion neurons interconnected into functional neuronal circuits, which constitute the human brain. Together, neurons establish bridges of communication through specialized cell junctions called synapses, where information is transmitted as electrical and/or chemical signals. Action potentials generated near the cell body arrive at the axon terminal inducing the opening of voltage-gated Ca^{2+} channels. Increase of intracellular Ca^{2+} triggers the release of neurotransmitters stored in the synaptic vesicles into the synaptic cleft, which will bind to specific ionotropic or metabotropic receptors on the postsynaptic membrane to conduct excitatory or inhibitory transmission by ion influx and activation of various signalling pathways. Therefore, variety in distinct presynaptic neurotransmitters and diverse postsynaptic specializations for downstream signalling, provide a vast combination for neuronal communication (Luscher & Huber, 2010; Malenka & Bear, 2004).

Glutamatergic synapses

Glutamate is the most common neurotransmitter in the central nervous system. It is released from the pre-synaptic terminal, binding and activating the following ionotropic postsynaptic receptors: alpha-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid receptors (AMPARs), kainite receptors and N-methyl-D-aspartate receptors (NMDARs) (Dingledine, Borges, Bowie, & Traynelis, 1999). These activated ligand-gated ion channels are permeable to Na^{+} and K+, and drive the postsynaptic neuron to depolarize (Lu & Malenka, 2012). Moreover, AMPARs mediate fast excitatory synaptic currents, and are crucial for the expression of various forms of long-lasting synaptic plasticity in the mammalian central nervous system (Malenka & Bear, 2004).

AMPA receptors

AMPARs can be composed by 4 different subunits: GluA1, GluA2, GluA3 and GluA4, which can form homomeric or heteromeric complexes. (Hollmann & Heinemann, 1994). Differences on subunit composition throughout development and synaptic plasticity alter channel molecular, biochemical, and physiological properties, and consequently fine-tune the mechanism expression via activation of distinct downstream pathways (Lohmann & Kessels, 2014; Malinow & Malenka, 2002; Santos, Carvalho, Caldeira, & Duarte, 2009).

All AMPAR subunits have an extracellular domain (N-terminal), four membrane-associated

hydrophobic domains and an intracellular domain (C-terminal) (Isaac, Ashby, & McBain, 2007). The C-terminal tail of each subunit co-assemble stoichiometrically with intracellular scaffolding or auxiliary proteins, including Stargazin, proximal N-ethylamide-sensitive fusion protein (NSF) / adaptor protein 2 (AP2) or other transmembrane AMPAR regulatory proteins (TARPs), which are responsible for stabilization or trafficking of AMPARs from/to the postsynaptic density (PSD) to the dendritic shaft, depending on their subunit composition, extracellular factors and intracellular signalling pathways (Isaac et al., 2007; Pandey, Rai, Gaur, & Prasad, 2015).

Widely expressed throughout neurons and in glia (Belachew & Gallo, 2004; Wisden & Seeburg, 1993), most AMPARs exist as heteromers, containing at least one subunit of GluA2 (Greger, Khatri, & Ziff, 2002; Wenthold, Petralia, & Niedzielski, 1996). Accordingly, expression of the functional heterotetrameric AMPA receptors and their number on the post-synaptic membrane is regulated either by transcriptional and RNA editing mechanisms or, alternatively, by their trafficking between endoplasmic reticulum (ER) to the post synaptic membrane via Golgi bodies (Dingledine et al., 1999; Isaac et al., 2007; Lu & Malenka, 2012; Pandey et al., 2015). In fact, the attention to GluA2-containig AMPARs has arise with the report that the GluA2 mRNA could be edited, rendering this subunit impermeable to divalent ions, such as Ca^{2+} (Liu & Zukin, 2007). The regulation of this process has an important role on development and on synaptic transmission (Z. Zhou, Hu, Passafaro, Xie, & Jia, 2011).

Notably, the current–voltage (I–V) relationship provides a biophysical signature for the different receptors, since AMPARs carry inward currents at negative potentials (-60 mV) and outward currents at positive potentials. Receptors that contain GluA2 experience a symmetrical current–voltage relationship, whereas nonlinear current-voltage ratio is seen on receptors that lack GluA2, for instance GluA1 homomeric or GluA1/3 heteromeric channels (Lu & Malenka, 2012) (Figure 1). For that reason, AMPARs comprising edited versions of GluA2 at the Q/R site present lower channel conductance than those lacking GluA2 subunits, since they have a glutamine residue in the pore region instead of the characteristic arginine, which allows high conductance for Na^{+} and even Ca^{2+}. Additionally, because endogenous polyamines are negatively charged, GluA2-lacking AMPARs are inhibited at positive potentials, which renders to these channels an inward-rectifying current–voltage relationship (i.e. inward currents are facilitated relatively to outward currents) (Huganir & Nicoll, 2013; Lu & Malenka, 2012).

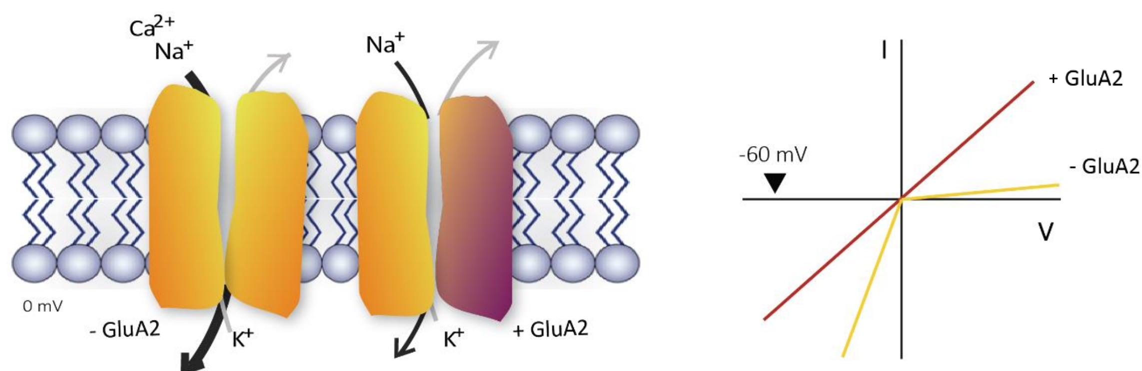

Figure 1| Current–voltage (I–V) relationship of AMPARs. A. Glutamate binding to AMPA receptors allows the entry of many sodium ions into the cell while only some potassium ions leave the neuron, causing a net depolarization of the membrane. **B.** When AMPARs comprise GluA2 subunit they present a linear I–V relationship, while AMPARs without GluA2 are inward-rectifying. Adapted from (Lu & Malenka, 2012).

AMPA receptors along neurodevelopment

Changes in AMPARs subunit composition not only underlie mechanisms behind synaptic plasticity but also occur along neurodevelopment (Figure 2) (Anggono & Huganir, 2012; Liu & Zukin, 2007; Pandey et al., 2015). Embryonic and immature postnatal hippocampus and cortex highly express GluA4, and low levels of activity are sufficient to promote the inclusion of GluA4 homomers into synapses located on dendritic shafts or small protrusions called filopodia (Zhu, Esteban, Hayashi, & Malinow, 2000). Alongside, dendrites and axons grow fast and establish new synaptic contacts, changing synaptic structure dramatically. Subsequently, increase on neuronal activity is accompanied by a decline on GluA4 expression (Akaneya, 2007), accompanied by an transient increase on alternatively spliced isoform of GluA2 ($GluA2_{long}$) mediated by either spontaneous activity or long-term potentiation (LTP). Like the GluA4 subunit, $GluA2_{long}$ is characterized by a long cytoplasmic tail that mediates synaptic strengthening during development (Kolleker et al., 2003). Later, GluA1, GluA2 and GluA3 start to rise in parallel and remaining high until the adult stage (Lohmann & Kessels, 2014; Wenthold et al., 1996). The number of synapses increases sharply during this period and the great majority of newly generated synapses are located on dendritic spines. Interestingly, RNA editing at the Q/R site, a unique feature of GluA2, arises during development, and its extent progresses with brain development reaching approximately 90% of total GluA2 (Lomeli et al., 1994)**.** Therefore, mature excitatory neurons of the hippocampus and cortex exhibit a smaller population of GluA1 homomers, GluA1/2 and GluA2/3 heteromers on equivalent amounts, since GluA2 homomers are unstable (Greger, Khatri, Kong, & Ziff, 2003; Greger et al., 2002; Wenthold et al., 1996). Indeed, studies have been demonstrated that the expression of GluA2 increases during hippocampus rodent development, both at the mRNA and protein levels (Pandey et al., 2015; Petralia et al., 1999).

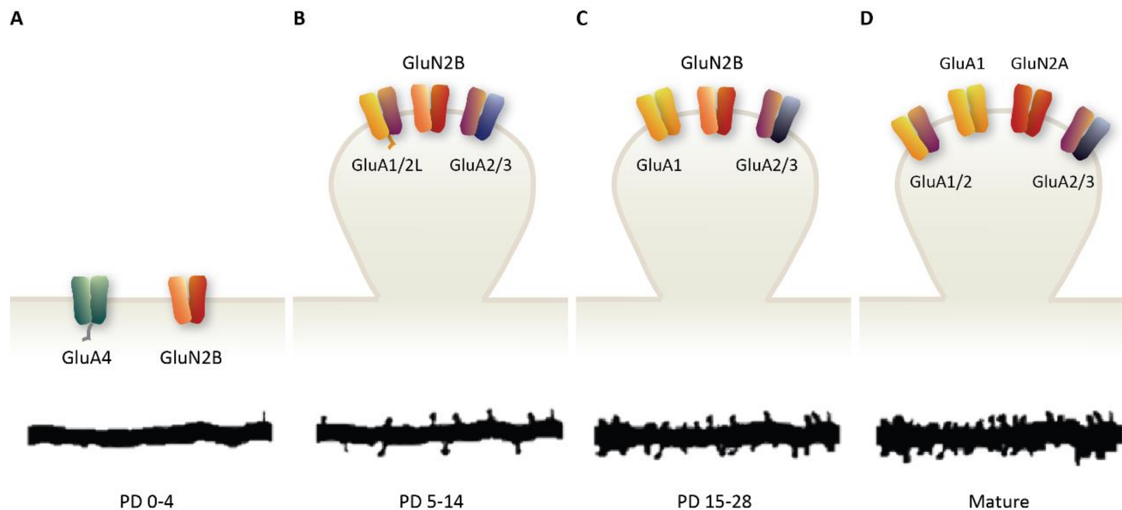

Figure 2| The structure and plasticity molecules of glutamatergic synapses at four fundamental stages of development. A. Between postnatal day 0 and 5 most synapses are located on the dendritic shaft, and contain AMPARs, which consist of GluA4, and NMDARs. **B.** Between postnatal days 5 and 14 synapses are still scarce but start occurring on spines, $GluA2_{long}$ is temporarily the dominant long-tailed AMPAR subunit, while short-tailed AMPAR subunits GluA2/3 also start being expressed. **C.** In the third and fourth weeks after birth spine numbers increase exponentially and the two dominant AMPARs are GluA1/2 and GluA2/3 heteromers. **D.** When the rodent is mature, spine numbers maximize, the developmental switch of GluN2B to GluN2A has occurred, with no alteration on AMPARs. Adapted from (Lohmann & Kessels, 2014).

AMPA receptors trafficking

Behind synaptic plasticity processes, namely LTP and long-term depression (LTD), relative rates of exocytosis and endocytosis at the postsynaptic membrane account for surface AMPARs content variations.

AMPARs are assembled in the ER and Golgi apparatus in the soma and are delivered into the dendrite via dynein– and kinesin-dependent vesicular transport on microtubule networks prior to their insertion to the plasma membrane mediated by SNARE (soluble NSF attachment receptor) proteins (Anggono & Huganir, 2012; Malinow & Malenka, 2002; Santos et al., 2009). After being incorporated at extrasynaptic sites of soma or dendrites, AMPARs undergo lateral diffusion until they reach the synapses, where they will be stabilized by PSD scaffolding proteins (Makino & Malinow, 2009). Although some studies support the hypothesis that AMPARs are first incorporated in extrasynaptic sites and subsequently translocated into synapses (Makino & Malinow, 2009; Y. Yang, Wang, Frerking, & Zhou, 2008), other authors claim a direct insertion of AMPARs at synaptic sites (Kennedy, Davison, Robinson, & Ehlers, 2010; Patterson, Szatmari, & Yasuda, 2010; Z. Wang et al., 2008). In the absence of electrical activity, synaptic GluA2/3 receptors are constitutively replaced through endosomal trafficking pathway, in a manner that maintains transmission constant (Malinow & Malenka, 2002; Santos et al., 2009). Hence, AMPARs undergo endocytosis mediated by clathrin from the plasma membrane to the early endosome. At this point receptors can either be delivered again into the plasma membrane directly or through recycling

endosome, or be driven to the degradation pathway mediated by late endosome (Anggono & Huganir, 2012). Albeit GluA2/3 receptor are continuously recycled, for instance, GluA1 and GluA2-containing AMPARs surface delivery is greatly enhanced or diminished by neuronal activity, respectively (Isaac et al., 2007; Lu & Malenka, 2012; Malinow & Malenka, 2002). Therefore, during LTD, constitutive AMPAR recycling outweighs the rate of AMPAR exocytosis, resulting in reduced synaptic AMPARs content (Ehlers, 2000). Nonetheless, total amount of AMPARs present in the cell might not be affected, since internalized receptors can be retained in intracellular compartment without lysosomal degradation. However, this effect is dependent on LTD stimulus paradigm (Malinow & Malenka, 2002; N. Zheng, Jeyifous, Munro, Montgomery, & Green, 2015). Conversely, under LTP, AMPARs are constantly delivered to the plasma membrane, by exocytosis at the synapse, to induce long-term maintenance of synaptic potentiation (Malinow & Malenka, 2002; N. Zheng et al., 2015).

Overall, AMPAR trafficking involves highly complex pathways tightly regulated by a series of orchestrated interactions with key intracellular regulatory molecules, which ultimately ensures synaptic function. As a consequence, binding disruption between AMPARs and its interacting proteins results in abnormal AMPAR trafficking outcome, impaired synaptic plasticity and deficits in learning and memory (Anggono & Huganir, 2012).

Synaptic plasticity

Neurons primary function is to receive, integrate and transmit information as an electrical or chemical signal from/to others neurons. In response to extrinsic stimuli, neurons are able to change and adapt the strength of their synapses, a mechanism in the basis of memory storage and learning processes (Pozo & Goda, 2010). Long lasting alterations in synaptic strength rely on the insertion or removal of AMPARs at the postsynaptic membrane, since these receptors mediate the majority of fast excitatory synaptic transmission (Malinow & Malenka, 2002; Nikkie F.M. Olde Loohuis et al., 2015). Hebbian plasticity, the most studied form of long lasting synaptic plasticity, comprises LTP and LTD. However, neuronal networks require their overall excitability to be maintained in a dynamic functional range. Therefore, homeostatic mechanisms are in place, through whit neurons can adjust the strength of their synapses up or down in order to stabilize firing (Turrigiano, 2008; Fernandes & Carvalho 2016).

Long-Term Potentiation

LTP is a widely accepted mechanism for synapse potentiation that mediates long-term information storage in the brain, by synaptic receptors enrichment and spine enlargement (Abbas, Villers, & Ris, 2015).

The induction of LTP occurs either by electrical stimulation with repetitive high-frequency (tetanic) presynaptic stimulation (HFS), or by chemically stimulation, through activation of metabotropic glutamate receptors (mGluRs) or through brain-derived neurotrophic factor/tropomyosin receptor kinase B (BDNF/TrkB) signalling (B. Ryan, Joilin, & Williams, 2015a). Some approaches has been successfully adopted and extensively used to investigate molecular and cellular events associated with LTP in hippocampal cultures, namely glutamate application or induction of glutamate release, calcium-evoked dendritic exocytosis, raise of intracellular cAMP concentration by the application of adenylyl cyclise activator and phosphodiestarase inhibitor (Molnár, 2011). Moreover, pre-exposure to the NMDAR antagonist (2R)-amino- 5-phosphonovaleric acid (AP-V) in Mg^{2+}-free medium favours activation of the NMDARs, even in the absence of NMDAR co-agonist glycine stimulation (Molnár, 2011). Accordingly, even a short period of synaptic activity can induce persistent changes on synaptic transmission lasting from minutes to days and often longer (Malinow & Malenka, 2002; Molnár, 2011).

Therefore, once LTP is induced in hippocampal neurons, glutamate is released from presynaptic terminal, activating AMPARs and NMDARs of postsynaptic membrane. After depolarization,

magnesium that blocks NMDARs is expelled allowing calcium entry in the cell, which in turn, activates calcium/calmodulin-dependent protein kinase II (CaMKII) pathway, resulting in trafficking and insertion of AMPARs into the postsynaptic membrane (Figure 3) (Bassani, Folci, Zapata, & Passafaro, 2013; Lisman, Yasuda, & Raghavachari, 2012; Lu & Malenka, 2012). CaMKII is activated through the binding of calcium to calmodulin, accompanied by autophosphorylation of Thr286 and Thr287 (Molnár, 2011). Subsequently, CaMKII diffuses from cytoplasm to PSD, where it binds to NR2B-containing NMDARs, locking the enzyme in an active state (Lisman et al., 2012) and phosphorylates GluA1 subunits of AMPARs at Ser831 (decreasing the energy required for structural conformational alterations that regulates the receptor conductance), inducing a potentiation of synaptic transmission through the increase in the average single channel conductance (Kristensen et al., 2011; Santos et al., 2009).

Simultaneously, CaMKII phosphorylates AMPAR-binding protein stargazin, which binds to PSD95, leading to the immobilization of AMPARs at the synapse (Bassani et al., 2013; Kauer & Malenka, 2007; Lisman et al., 2012). Indeed, the primary subtype of AMPARs that is inserted into synapses during LTP seems to be heteromeric GluA1/GluA2 receptors (Lu & Malenka, 2012), although the switch of GluA2-containing AMPARs in the synapse for GluA2-lacking AMPARs has also been correlated with synaptic potentiation (Liu & Zukin, 2007). In fact, findings suggest that during the very early phase of LTP, a transient insertion of GluA2-lacking AMPARs, mainly GluA1 homomers, at the synapse is required for the maintenance of LTP (Isaac et al., 2007; Plant et al., 2006). Later (~20 min after stimulus), synaptic strength is stabilized by replacement of GluR1 homomers with GluA2-containing AMPARs, in a mechanism independent of NMDAR activation and driven by Ca^{2+} influx through GluR2-lacking AMPARs (Isaac et al., 2007; Santos et al., 2009). Therefore, the rapid AMPARs accumulation and immobilization at synapses is the result of both their exocytosis and stabilization at the postsynaptic density, due to the trafficking of AMPARs from extrasynaptic sites to the synapse (Makino & Malinow, 2009; Molnár, 2011). Overall, these alterations lead to an increase of miniature excitatory postsynaptic currents (mEPSCs). However, it has been demonstrated that this mechanism only explains short-term synapse strengthening and only lasts for few hours (Joilin, Guévremont, Ryan, Claudianos, & Cristino, 2014; Lisman et al., 2012; B. Ryan et al., 2015a), which prompted the proposal of a model where LTP comprise two temporally and mechanistically different phases – early-LTP (E-LTP) and late-LTP (L-LTP), albeit some authors divide the second one in two additional parts (Abbas et al., 2015; Fonseca, Nägerl, & Bonhoeffer, 2006; B. Ryan et al., 2015a). Contrary to the early phase described above, the latter two phases require *de novo* protein synthesis, namely synthesis of AMPARs subunits, in order to maintain LTP for a longer period of time.

In the initial stage of L-LTP, protein synthesis takes place locally at the synapse and gene transcription is not required (B. Ryan et al., 2015a; Tsokas et al., 2005). However, the dendrite by

itself does not have the ability to maintain LTP for long period of time, relying only on local mRNA translation (B. Ryan et al., 2015a). Based on this data, it was proposed that new gene transcription as well as local translation must occur for maintaining LTP. For this reason, at the end phase of L-LTP, both constitutive (e.g. cAMP response element-binding protein (CREB)) and inducible transcription factors (e.g. early growth factor-1) are activated (Joilin et al., 2014; B. Ryan et al., 2015a).

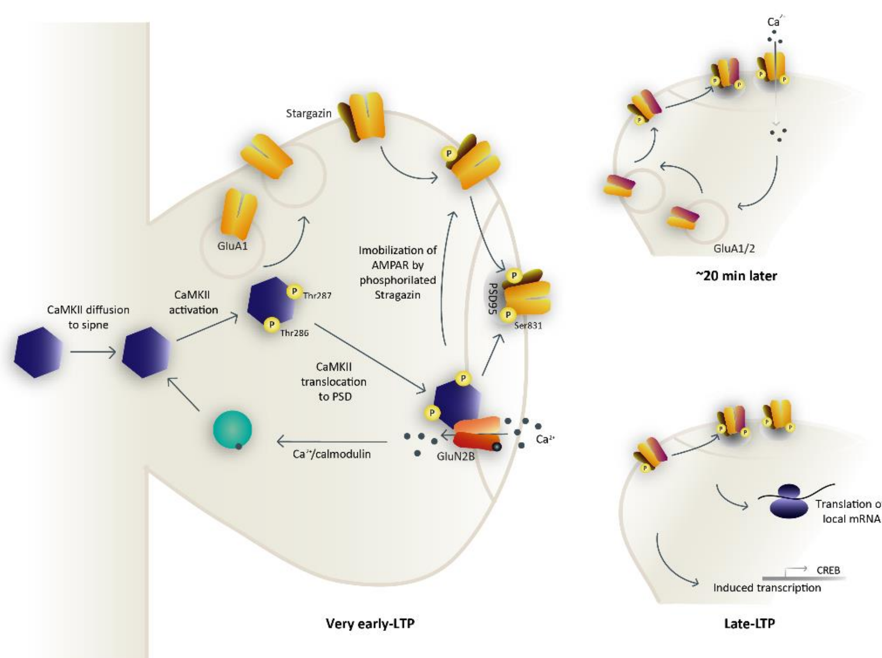

Figure 3| Mechanisms for surface AMPARs increase at synapse during long-term potentiation. Calcium entry through NMDA-type glutamate receptors (NMDARs) leads to the activation of calmodulin. Calmodulin activates calcium/calmodulin-dependent protein kinase II (CaMKII), which then translocates to the postsynaptic density (PSD), where it enhances AMPA-type glutamate receptor (AMPAR)-mediated transmission in two ways. First, CaMKII phosphorylates AMPAR glutamate receptor 1 (GluR1) subunits at S831, which leads to an increase in the average conductance of such channels. Second, CaMKII phosphorylates the AMPAR-binding protein stargazin, which causes stargazin to bind PSD95, thereby increasing the number of AMPARs at the synapse. CaMKII activity, perhaps with other calcium-dependent processes, stimulates the fusion of vesicles containing AMPARs with the plasma membrane, increasing the extrasynaptic concentration of such channels. 20 min after stimulus, synaptic strength is stabilized by replacement of GluR1 homomers with GluA2-containing AMPARs, in a mechanism driven by Ca2+ influx through GluR2-lacking AMPARs. Later, both constitutive CREB and inducible transcription factors are activated. Adapted from (Lisman et al., 2012).

Upon LTP induction, besides the insertion of additional receptors on postsynaptic sites, structural alterations in dendritic spines also take place, due to stabilization of actin filaments, prompting an increase in spine size (Bosch & Hayashi, 2012). Accordingly, Arc, an immediate early gene, has been reported to orchestrate processes related to LTP consolidation, namely AMPAR trafficking, PSD remodelling and spine enlargement (by the regulation of actin dynamics) (Bramham et al., 2010). In addition to alterations of synapse ultrastructure, new spines can appear within minutes after LTP induction (Lisman et al., 2012; Lu & Malenka, 2012).

Hence, together nuclear transcription, local dendritic and somatic protein synthesis are key mechanisms to maintain functional and structural alterations triggered by LTP.

Long-Term Depression

Accumulated evidence supports a crucial role of LTD in learning, memory and cognition, tightly regulating AMPAR function at mature synapses (Collingridge, Peineau, Howland, & Wang, 2010).

Contrary to LTP, LTD is proposed to result from sustained entry of low levels of calcium into postsynaptic neurons, in response to low-frequency glutamate stimulation, since most studies focus on LTD forms that are triggered by synaptic activation of either NMDARs or mGluRs in excitatory neurons (Bassani et al., 2013; Collingridge et al., 2010). Interestingly, AMPAR endocytosis could be also triggered via an insulin-activated signalling pathway (Beattie et al., 2000; Man et al., 2000).

NMDAR-dependent LTD

NMDAR-dependent LTD is characterized by the activation of NMDA receptors, allowing a modest calcium influx through these channels, in lower levels than the ones required for LTP (Figure 4). As a consequence, protein phophatase 1 (PP1) and protein phosphatase calcineurin are activated, via a serine-threonine protein phosphatase cascade (Beattie et al., 2000; Malenka & Bear, 2004). These enzymes dephosphorylate several downstream targets, including PSD proteins and proteins that are part of the endocytic complex. Even though the exact mechanism are still unknown (Bassani et al., 2013; Huganir & Nicoll, 2013), several lines of evidence indicate that the interaction between calcineurin and dynamin 1 functions as a calcium sensor for endocytosis, that allows complex binds to amphiphysin 1. As a consequence, this complex is then delivered to the endocytic protein complex, allowing calcineurin to dephosphorylate endocytic proteins, which in turn promotes the assembly and function of the endocytic complex (Beattie et al., 2000). Therefore, following substrate dephosphorylation, a rapid internalization of postsynaptic AMPARs and downregulation of NMDARs is observed, reducing strength and synaptic transmission (Bassani et al., 2013; Huganir & Nicoll, 2013; Malenka & Bear, 2004).

During this process, GluA2 appears to be a crucial mediator of AMPAR removal from the synapse. In more detail, GluA2 loses the affinity for NSF, an ATPase known to play an important role in membrane fusion, allowing the binding with clathrin adaptor protein AP2, which in turn, promotes assembly of the clathrin coat resulting in AMPARs endocytosis. Moreover, prior to internalization, synaptic AMPARs diffuse laterally to endocytic sites, adjacent to the PSD (Bassani et al., 2013; Malenka & Bear, 2004; Santos et al., 2009). There are also several proteins interacting with GluA2, such as GRIP1/2 (Glutamate receptor-interacting protein 1/2) and PICK1 (Protein Interacting with C Kinase 1), which are known regulators of AMPAR trafficking in LTD. The affinity of GluA2 for

GRIP1/2 or PICK1 is regulated by the phosphorylation status of Ser880. During LTD, in response to Ca^{2+} influx, Protein kinase C (PKC) phosphorylates GluA2 at Ser880, which raises the GluA2 affinity for PICK1 and, in turn leads to AMPARs endocytosis (Bassani et al., 2013; Santos et al., 2009). Not only Ser880 phosphorylation, but other posttranscriptional modification of the AMPAR C-terminal domains are described to trigger GluA2-containing AMPARs internalization, such as Tyr876 dephosphorylation, which induces BRAG2-mediated Arf6 activation, a process critical for targeted receptor endocytosis (Scholz et al., 2010).

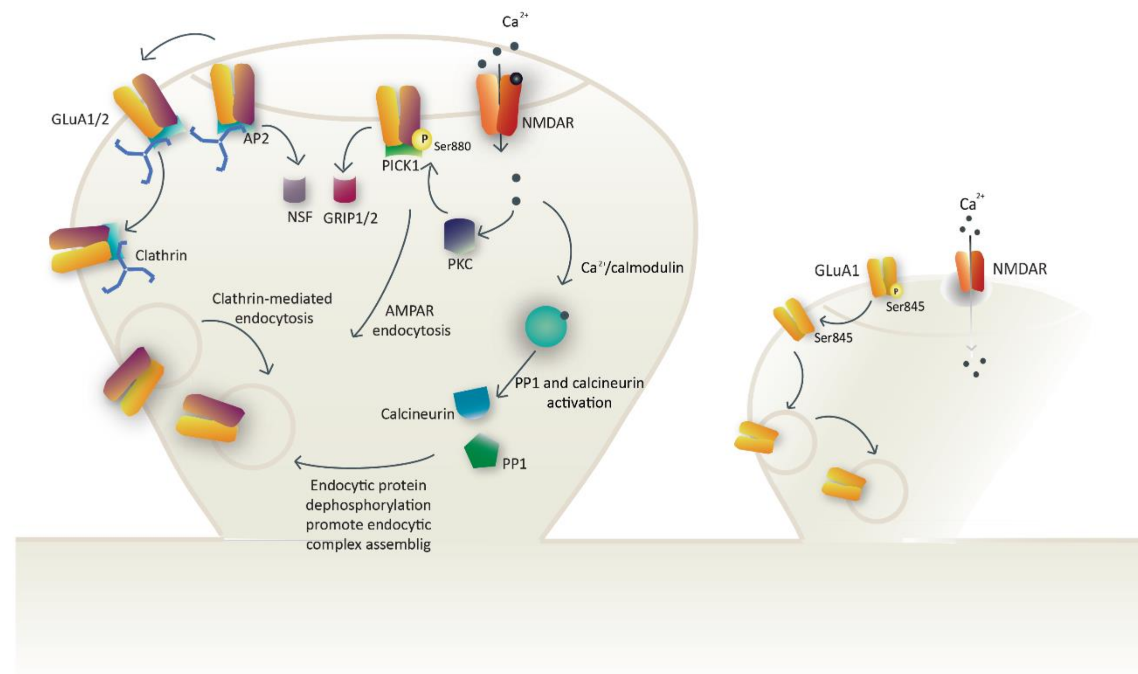

Figure 4| Pathways that underlie long-termed depression dependent of NMDAR activation. Calmodulin detects modest increases in calcium levels, triggered by NMDAR activation, activating protein phophatase 1 (PP1) and protein phosphatase calcineurin. Calcineurn dephosphorylates endocytic proteins, which in turn promotes the assembly and function of the endocytic complex, resulting on a rapid internalization of postsynaptic AMPARs. Simultaneously, GluA2 loses the affinity for NSF allowing the binding with clathrin adaptor protein AP2, which ultimately after lateral diffusion, promotes assembly of the clathrin coat resulting in AMPARs endocytosis. Additionally, calcium influx leads to PKC phosphorylation of Ser880 in the GluA2 subunit. Subsequently, GluA2 binds to PICK1, which triggers AMPAR internalization. At the same time, GluA1 subunit is dephosphorylated on Ser845, resulting in a decrease on AMPA open channel probability and consequent endocytosis. Based on (Bassani et al., 2013).

In addition, studies have reported that dephosphorylation of both PKA and PKC substrates, including GluA1 dephosphorylation, have also critical roles in LTD phenomena (H. K. Lee, Kameyama, Huganir, & Bear, 1998). Conversely to LTP, LTD was found to be associated with selective dephosphorylation of Ser845, resulting in a decrease probability of AMPA open channel, without any change in Ser831 (Banke et al., 2000; H. K. Lee et al., 1998). In fact, internalized GluA1 AMPARs present Ser845 dephosphorylated accordingly Ehlers's work (Ehlers, 2000).

Notably, the existence of two functionally distinct populations of AMPARs at the cell surface is

now accepted to explain different mechanisms of AMPARs internalization. Thus, on the one hand, the non-stabilized receptors, more susceptible to diffusion into clathrin-coated pits, do not require phosphatase activity above basal levels to be endocytosed. On the other hand, internalization of AMPARs that are stabilized at the synaptic surface may require calcium-dependent activation of calcineurin, which destabilizes protein–protein interactions between receptors and PDZ-domain-containing proteins, resulting ultimately on endocytic machinery enhancement (Beattie et al., 2000).

Recent provocative experiments have proposed a "metabotropic" action for NMDARs under high-frequency synaptic stimulation, whereby a conformational change in the receptor, independent of ion flux, triggers downstream signalling pathways resulting in LTD (Kessels, Nabavi, & Malinow, 2013).

mGluR-dependent LTD

An analogous form of LTD, mediated by mGluRs activation, has been characterized at cerebellar parallel fibre–purkinje cell and hippocampal synapses (Kauer & Malenka, 2007; Lu & Malenka, 2012). Unlike NMDAR-LTD, mGluR-depend LTD does not require NMDAR activation and postsynaptic protein phosphatases; instead, it is induced by simultaneous activation of postsynaptic mGluR1 and mGluR5 receptors and voltage-gated calcium channels (Figure 5). Thus, brief mGluR activation could be achieved either by pharmacological application of the Gp1 mGluR agonist R,S-dihydroxyphenylglycine (DHPG) or by prolonged low frequency synaptic stimulation (Luscher & Huber, 2010). Consequently, LTD induction and subsequent AMPAR endocytosis follows a non-canonical pathway that relies on tyrosine dephosphorylation and tyrosine phosphatase striatal-enriched tyrosine phosphatase (STEP), in a PKC-independent manner (Luscher & Huber, 2010). Therefore, activation of mGluR1/5 elicits STEP-mediated dephosphorylation of GluA2 on Try residues triggering AMPARs endocytosis. Contributing for this model, TACE (tumor necrosis factor-a-converting enzyme), a matrix metalloproteinase, has been described to cleave the neuronal pentraxin receptor (NPR), leading the NPR ectodomain to "capture" AMPARs through extracellular interactions, which in turn stimulates their endocytosis (Cho et al., 2008). Interestingly, although under some circumstances this process can occur independently of protein synthesis, generally *de novo* protein synthesis is required during mGluR-LTD, which indicates an important role for locally synthesized proteins on regulation of AMPAR endocytosis and/or trafficking after endocytosis, although the mechanisms affected by this remain largely unclear (Greenough et al., 2001; Huber, Kayser, & Bear, 2000; Luscher & Huber, 2010). Among a vast pool of newly synthetized proteins, fragile X mental retardation protein (FMRP) and activity-regulated cytoskeletal associated protein (Arc) seems to be implicated on LTD induction and maintenance (Greenough et al., 2001; Waung & Huber, 2009) . In support of this data, rapid synthesis of Arc protein locally occurs to maintain LTD by increasing GluA1 endocytosis rate, with

a requirement of existing Arc protein for the LTD-induction phase. Indeed, Arc levels are described to remain elevated for at least 1 h (Waung & Huber, 2009), since due to interaction with endophilin2/3 and dynamin, Arc acts as a scaffold localizing endocytosis machinery, culminating on modulation of receptor internalization (Luscher & Huber, 2010; Waung & Huber, 2009).

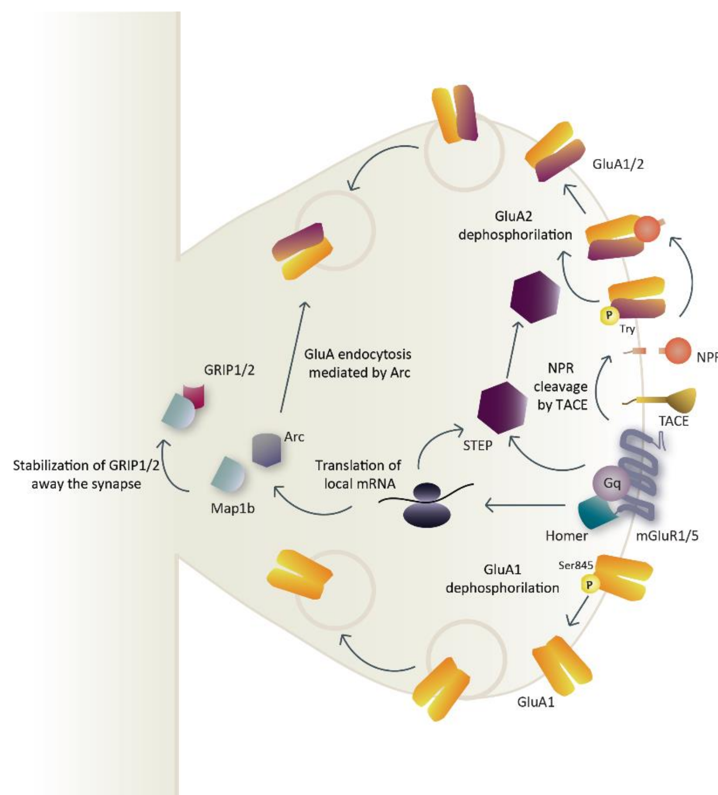

Figure 5| Pathways that underlie long-termed depression mediate by mGluR activation, in the hippocampus. In hippocampal CA1 neurons, brief activation of mGluR1/5 triggers rapid endocytosis of AMPARs through TACE-mediated intramembrane cleavage of NPR. mGluR-stimulated AMPAR endocytosis requires activity of the Tyr phosphatase STEP as well as existing Arc protein. mGluRs also trigger translation of proteins through activation of translation initiation, as well as dephosphorylation of the RNA binding protein, FMRP. Known proteins whose synthesis is stimulated by mGluRs and that play a role in mGluR-LTD include STEP, MAP1b and Arc. These proteins are known to regulate and/or stimulate AMPAR endocytosis (Adapted from Luscher & Huber, 2010).

Together with Arc, synthesis of new STEP may promote the maintenance of GluA2 in a Tyr dephosphorylated state. Likewise, microtubule associated protein 1b (MAP1b) synthesis during LTD stabilizes GluA scaffold protein GRIP1 away from the synapse, ending up destabilizing GluA surface expression. Overall, mGluRs stimulate a coordinated translation of proteins that together reduce surface AMPAR expression causing a persistent synaptic depression (Kauer & Malenka, 2007; Luscher & Huber, 2010; Malenka & Bear, 2004; Santos et al., 2009; Waung & Huber, 2009).

Defining the signalling pathways by which mGluRs control translation provides insight into the plasticity mechanisms. In view of that, both translation initiation and elongation are under mGluR regulation via ERK-MAPK and PI3K-mTOR pathways. On the end, metabotropic receptor activation, through a Homer scaffold, concurrently stimulates translation initiation while slightly

inhibiting elongation, which coordinates translational activation of specific mRNAs required for LTD, namely Arc and MAP1b (Luscher & Huber, 2010; Waung & Huber, 2009).

Conversely to LTP, alongside the decreased number of surface AMPARs, LTD enhances shrinkage of dendritic spines and even their disappearance (Lu & Malenka, 2012). Indeed, NMDA application causes the loss of F-actin puncta in dendritic spines (Halpain, Hipolito, & Saffer, 1998). However, NMDA-induced actin breakdown blockade has no effects on AMPAR endocytosis, indicating that, interestingly, dramatic rearrangement of the spine's actin cytoskeleton is not required for the local trafficking of endocytic vesicles that contain AMPARs (Beattie et al., 2000).

At the end, all mechanisms behind LTD are reflected in a decrease in AMPA-mediated mEPSC, consistent with evidence of reduced expression of postsynaptic AMPARs (Beattie et al., 2000; J. Zhang, Yang, Li, Cao, & Xu, 2005), with parallel depression of NMDAR EPSCs and NMDA-evoked responses under mGluR-activation (Snyder et al., 2001), ultimately leading to a decrease in synaptic strength.

Homeostatic plasticity.

Hebbian plasticity is characterized by positive feedback mechanisms that leave the synapse more suitable to undergo further potentiation or depression, due to the reduction or increase of the depolarization threshold. Thus, in order to prevent neuronal activity to reach a saturated state and to keep it in an optimal set-point range, a compensatory system takes place – homeostatic plasticity. Synaptic scaling is form of homeostatic plasticity that globally scales the strength of a neuron's synapses up or down in order to stabilize neuronal firing (Cohen, Lee, Chen, Li, & Fields, 2011; Pozo & Goda, 2010; Turrigiano, 2008; Fenandes & Carvalho 2016).

Neurons can sense the level of network activity and scale up or down the synaptic strength and connectivity accordingly, in a multiplicative manner. Thus, relative differences in synaptic strength between synapses are maintained, avoiding disruption of information storage or processing mechanisms that rely on those differences (Bassani et al., 2013; Pozo & Goda, 2010; Turrigiano, 2008; Fenandes & Carvalho 2016). In fact, neurons can detect alterations in their own firing rates through the amount of somatic calcium and consequently, change a set of calcium-dependent sensors and transcription factors, in order to regulate AMPARs trafficking to increase or decrease the receptor accumulation on the surface (Figure 6) (Turrigiano, 2008).

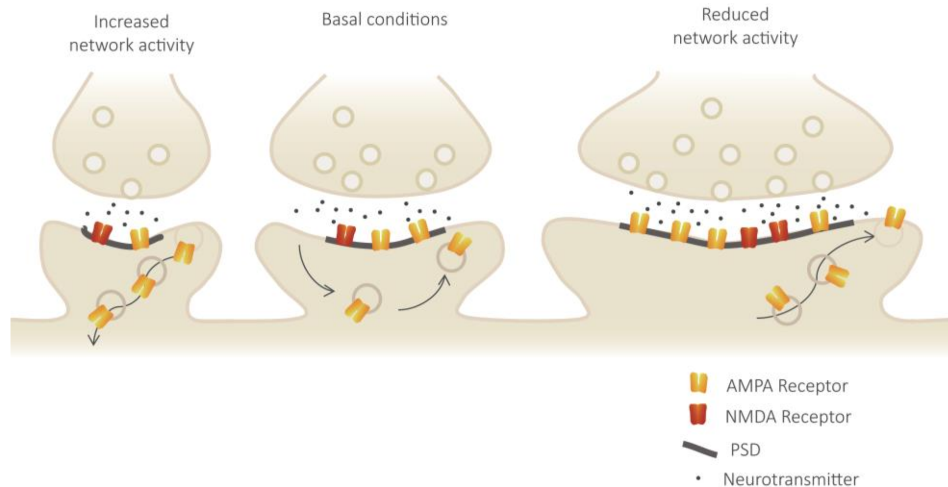

Figure 6| Homeostatic synaptic plasticity at the excitatory synapse. At basal conditions, synaptic transmission is mediated by the release of neurotransmitters from the presynaptic terminal and subsequent activation of receptors at the postsynaptic cell. Prolonged reduced activity enhances the recycling of vesicles, the number of docked vesicles and the release probability. Postsynaptically, additional neurotransmitter receptors are incorporated at the synapse. Chronically increased network activity decreases release probability and leads to a reduction in the number of postsynaptic receptors. (Adapted from Pozo & Goda, 2010).

However, some studies *in vivo* and in organotypic slices reveal that synaptic scaling is not always uniform and suggest that changes in network activity do not affect equally all synaptic inputs onto a single neuron, inversely to the uniform multiplicative alterations reported in dissociated cultures (Pozo & Goda, 2010; Turrigiano, 2008). Moreover, synaptic changes were found to be more potentiated at larger synapses and restricted to small subsets of synapses along the dendritic branch (Branco, Staras, Darcy, & Goda, 2008; Hou, Zhang, Jarzylo, Huganir, & Man, 2008; Ju et al., 2004; Minerbi et al., 2009; Turrigiano, 2008).

Additionally, firing rates are kept stable by balancing inward and outward voltage-dependent conductances that determine firing properties (intrinsic excitability), regulating inhibitory and/or excitatory synaptic strength, or synapse number, or adjusting the ease with which other forms of plasticity can be induced (Turrigiano, 2008).

Given the role of local glutamate signaling in homeostatic plasticity, two distinct plasticity mechanisms have been suggested.

- A global mechanism triggered by blockage of postsynaptic firing while presynaptic and network activity are intact, via a mechanism that results in increased accumulation of both GluA1 and GluA2 subunits of AMPARs in the dendrites (Pozo & Goda, 2010; Turrigiano, 2008).
- A local mechanism that is generated when action potentials and NMDARs are blocked simultaneously, leading to a local increase in synaptic GluA1 accumulation that requires

local dendritic protein synthesis (Pozo & Goda, 2010; Turrigiano, 2008).

Thus, to induce synaptic scaling postsynaptically, standard paradigms comprising blockade or enhancement of network activity in culture and sensory deprivation in live studies have been applied (Turrigiano, 2008). In particular, a study noticed similar effects of somatic block of spikes and of global block of network activity with tetrodotoxin (TTX), suggesting that blocking postsynaptic spikes is sufficient to induce full-blown scaling up of synaptic strengths, highlighting the distinct role for somatic and dendritic depolarization on these processes (Ibata, Sun, & Turrigiano, 2008). On other hand, a presynaptic form of homeostatic plasticity is achieved by expressing inwardly rectifying potassium channels to chronically hyperpolarize individual neurons (Turrigiano, 2008). As a consequence, presynaptic expression of homeostatic changes is not limited to targeting the synaptic vesicle cycle and the efficacy of neurotransmitter release, but alterations in vesicular glutamate transporter VGluT1 expression could lead bidirectional homeostatic adaptations (Pozo & Goda, 2010).

Inversely to local change in synaptic signalling transmission characteristic in Hebbian mechanisms, a global mechanism to induce a widespread homeostatic plasticity in network activity has been proposed, relying on activity-dependent release of soluble factors by many neurons or glia simultaneously (Turrigiano, 2008). Hence, described molecules include BDNF that promotes inhibitory synaptic activity under excitatory conditions; tumor necrosis factor α (TNFα) released by glial cells upon activity deprivation, promotes the delivery of GluA2-lacking AMPARs to cell surface and removal of gamma-aminobutyric acid A ($GABA_A$) receptors; finally, retinoic acid rapidly scales up AMPARs triggering GluA1 local protein synthesis, when neuronal activity is blocked (Sutton & Schuman, 2006).

Furthermore, local changes at individual synapses could also be mediated by a pathway dependent of synapse adhesion proteins. Accordingly, cells adhesion molecules, such as integrins, could regulate synaptic strength by acting on AMPARs stabilization, through communication between extracellular changes and intracellular signalling pathways or actin scaffolds. Therefore, homophilic or heterophilic adhesion proteins coordinate changes in neurotransmitter release and postsynaptic receptors during homeostatic adaptation (Bassani et al., 2013; Pozo & Goda, 2010; Turrigiano, 2008).

Moreover, PSD scaffolding proteins, including PICK1 and MAGUKs; intracellular signalling molecules, for instance, CaMKs, Polo Like Kinase 2 (PLK2), PI3K-Akt; proteins related to activity-induced gene expression, Arc; a protein involved in SUMOylation (SENP1 Sentrin-specific protease 1) as well as the ubiquitin proteasome system has been noticed to be implicated in the scales up or down AMPARs at synapse (Bassani et al., 2013; Pozo & Goda, 2010; Turrigiano, 2008). In the same way, posttranscriptional modifications of AMPARs partners in response to

chronic activity blockade, namely stargazin phosphorylation, seem to be essential for scaling up, regulating AMPARs trafficking (Louros, Hooks, Litvina, Carvalho, & Chen, 2014).

microRNA – The basis

MicroRNAs (miRNAs) are small non-coding RNAs with about 18-22 nucleotides (Ha & Kim, 2014). These molecules constitute an important post-transcriptional regulatory mechanism, either by inhibiting protein synthesis or by targeting mRNAs for degradation. Although not every interaction has physiological relevance, each individual miRNA usually targets up to a few hundred different RNAs, establishing a vast regulatory RNA network that allows a flexible control of mRNA expression. Thus, it is not surprising that the miRNA regulation takes place at multiple steps, including transcription, processing, loading onto argonaute proteins (AGO proteins) and miRNA turnover (Ha & Kim, 2014; G. Schratt, 2009).

miRNA biogenesis

In humans, some miRNAs are encoded by exonic regions, but the majority of canonical miRNAs are encoded by non-coding regions or introns of coding transcripts. Several miRNA genes can be encoded as a cluster that comprises 2-7 genes close to each other, constituting a polycistronic transcription unit controlled by a single and common promoter. This allows miRNA expression at levels that could not be achieved by transcription from a single miRNA gene (O'Carroll & Schaefer, 2012). However, there is also the possibility that individual promoters drive transcription of each miRNA gene, although precise localization of miRNA promoters remains to be mapped for the majority of miRNA-encoding genes. In most of these cases, single miRNA genes were located on different chromosomes, providing a mechanism that ensures the expression of essential miRNAs when one of the miRNA-encoding alleles suffers deletions (O'Carroll & Schaefer, 2012). For instance, three highly abundant neuron-specific miRNAs – miRNA-124, miRNA-7 and miRNA-9 – are each encoded in three different clusters in humans (Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006). Moreover, individual miRNAs are additionally regulated at post-transcriptional level (Ha & Kim, 2014; Y. Lee, Jeon, Lee, Kim, & Kim, 2002).

The current model of miRNA biogenesis (Figure 7) postulates that a miRNA gene is transcribed by RNA polymerase II (Pol II), giving rise to a long primary miRNA (pri-miRNA) with a local hairpin structure where mature miRNA sequences are embedded (Ha & Kim, 2014). The resulting transcript is structured in upper and lower stem, a terminal loop, apical and basal junctions and single-stranded RNA segments at both the 5' and 3' sides (Figure 8). Following transcription, microprocessor complex recognizes and cleaves the pri-miRNA. This complex comprises nuclear

ribonuclease III (RNaseIII) Drosha, necessary for cleavage of RNA stem-loop and its co-factor DGCR8, which is responsible for recognizing the pri-miRNA. As a result, small hairpin-shaped RNA – precursor miRNA (pre-miRNA) – with 70–100 nucleotides is produced (Bartel, Lee, & Feinbaum, 2004; Ha & Kim, 2014).

Thereafter, the pre-miRNA binds to Ran-GTP and exportin 5, forming a transporter complex, which allows the translocation of pre-miRNA to the cytoplasm, where its maturation can be completed (Bartel et al., 2004; Ha & Kim, 2014; Kosik, 2006). Once the transport is completed, this complex disassembles, due to GTP hydrolysis, releasing the pre-miRNA into the cytoplasm. Nonetheless, a recent study described that the exportin1-dependent transport is the dominant pathway for some non-cannonical pre-miRNA, namely for m^{7}G-capped pre-miRNAs (Xie et al., 2013).

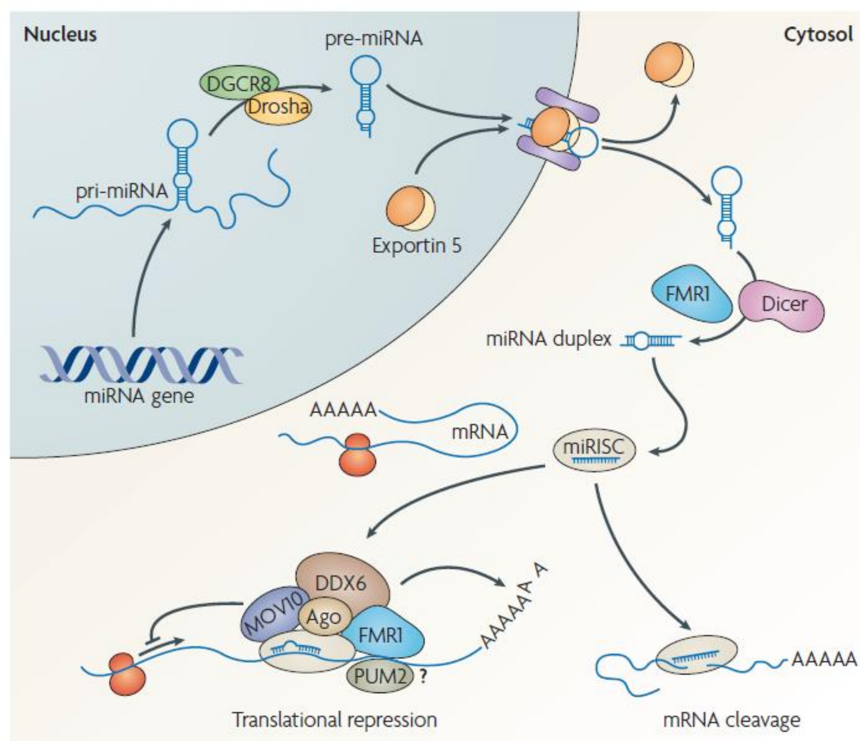

Figure 7| Canonical miRNA biogenesis. miRNAs are transcribed by RNA polymerase II giving rise a long primary miRNA that has a local hairpin structure where mature miRNA sequences are embedded. Following transcription, DGCR8 recognizes the pri-miRNA and RNaseIII Drosha cleaves it. As a result, the pre-miRNA is produced, with 70–100 nucleotides and with a small hairpin. Thereafter, pre-miRNA is actively transported from the nucleus to the cytoplasm by Ran-GTP and exportins. In the cytosol, pre-miRNA is cleaved by Dicer at the base of the stem loop in order to process the other end of mature miRNA. Thus, the cleavage product is a siRNA-like imperfect duplex that comprises the mature miRNA. Afterward, the RNA duplex, with about 22 nucleotides, is loaded onto AGO proteins, depending on ATP, to form the precursor form of RNA-induced silencing complex. Subsequently, RNA duplex undergoes unwinding and one of the strands is removed, generating a mature RISC. Consequently, the released passenger strand is quickly degraded. Then, RISC is delivery into P body that comprises untranslated mRNAs. Therefore, the miRNA target the mRNA depending on the degree of complementarity between 3'UTR of target mRNA and the seed sequence of miRNA, protein synthesis is destabilized by imperfect binding, whereas mRNA cleavage is mediated by high complementary sequences (G. Schratt, 2009).

Once in the cytoplasm, Dicer cleaves the miRNA in order to process the other end of the miRNA, since the first one is defined by nuclear cut of Drosha (Bartel et al., 2004). Hence, in mammalian and flies, Dicer is an RNaseIII-type endonuclease, which recognizes the double-stranded portion of the pre-miRNA, and it has specific affinity for 5'phosphate and 3'overhang at the base of the stem loop. Subsequently, the enzyme cleaves both strands of the duplex, 22 nucleotides away from the 5´ and at 21–25 nucleotides of distance from the 3´ end of the terminus, releasing a small RNA duplex (Figure 8) (Bartel et al., 2004; Ha & Kim, 2014). Nonetheless, the binding of Dicer only takes place when the end is thermodynamically unstable and for example it does not occur when the pre-miRNA terminal is binding strongly like through G-C base pairs (Ha & Kim, 2014). Moreover, its N-terminal helicase domain interacts with terminal loop of pre-miRNA, facilitating the recognition. Human Dicer has a structure arrangement that allows it to occupy simultaneously 5' and 3' ends of the pre-miRNA, when RNA has two-nucleotide-long 3´ overhang structure, which explains the preference for two-long 3'overhang initially generated by Drosha (Ha & Kim, 2014). The resultant cleavage product is a siRNA-like imperfect duplex that comprises the mature miRNA and similar-sized fragment derived from the opposing arm of the pre-miRNA.

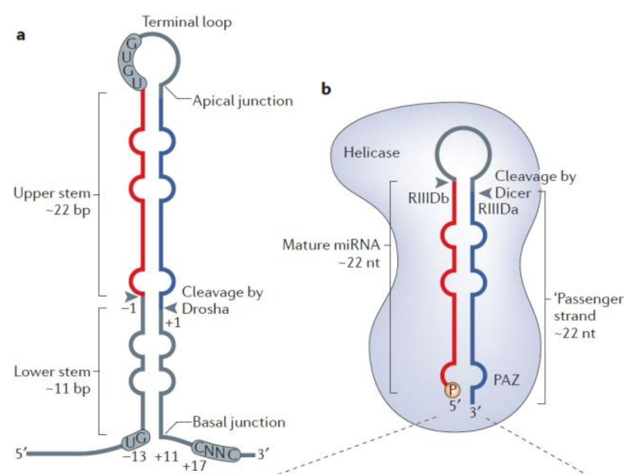

Figure 8| Structure of pri-microRNA (left) and pre-microRNA (right) highlighting respective cleavage sites by Drosha and Dicer, respectively (Minju Ha and V. Narry Kim, 2014).

After processing, the RNA duplex is loaded by AGO proteins, according to its intrinsic structural properties, which prompts the formation of a precursor form of RNA-induced silencing complex (RISC). Four AGO proteins (AGO1 – AGO4) are implicated in miRNA or in both miRNA and small interfering RNA (siRNA) pathways, however only siRNA binds to AGO2 to repress it (Filipowicz, Bhattacharyya, & Sonenberg, 2008; Ha & Kim, 2014). In flies, 5'nucleotide determine the load of small RNAs into AGO proteins, since miRNAs that present 5'uracil preferentially bind to AGO1 while miRNAs with 5'cytosine are sorted into AGO2. Contrariwise, in humans, all four

human AGO proteins can incorporate both siRNA and miRNA duplexes, with a preference for small RNA duplexes with central mismatches (nucleotide positions 8–11). Additionally, small-RNA duplexes loading into RISC is an active process greatly facilitated by ATP (Yoda et al., 2010).

Subsequently, miRNA assembly into the RISC involves unwinding of RNA duplex and removal of one of the strands, in order to generate a mature RISC that provides sequence to direct it to its target. Hence, the selection of which strand is release – passenger strand – and which remains into RISC – guide strand – lies on the thermodynamic stability of the two ends of duplex (Bartel et al., 2004). Therefore, the strand with relatively unstable terminus at 5′ side and with an uracil at nucleotide position 1 is typically selected as the guide strand. The unwinding of miRNA duplex can occur either through a cleavage-depend mechanism or through a cleavage-independent mechanism being the cleavage-independent a more common process, which is promoted by the mismatches at nucleotide positions 2–8 and 12–15 in the guide strand (Kawamata, Seitz, & Tomari, 2009; Yoda et al., 2010). The released passenger strand can either be quickly degraded or promote mRNA silencing, although with less potency and abundance than the guide strand (Ha & Kim, 2014). Additionally to AGO proteins, other proteins are known to be part of the RISC – such as MOV10, FMRP, DDX6 –functioning as RISC assembly and regulatory factors, or as effectors mediating repressive RISC functions (Filipowicz et al., 2008).

However, an alternative mechanisms for the biogenesis of about 1% of conserved miRNA or miRNA-like small RNAs, independently of Dicer or Drosha, has already been reported in vertebrates. A non-canonical pathway was first identified in *Drosophila*, during *miRNAtron* (pre-miRNAs/intron) production, in which debranched short hairpins introns of mRNA mimic the structural features of pre-miRNAs, undergoing the miRNA processing pathway without Drosha-mediated cleavage. As Drosha cleavage is bypassed, the pre-miRNA is formed through mRNA splicing (Ha & Kim, 2014; Ruby, Jan, & Bartel, 2007). Opposite to *Drosophila*, in mammals only few miRNAtrons have been proved, namely miRNA-877, miRNA-702, and miRNA-1124–miRNA-1141 (Berezikov, Chung, Willis, Cuppen, & Lai, 2007). In addition, some small RNAs may originate from other non-coding RNAs, such as tRNAs or tRNA-like precursors, small nucleolar RNAs (snoRNAs) or small nuclear RNA-like viral RNAs, in the absence of Drosha-mediated processing. Although the biogenesis of these small RNAs is Drosha independent, it is still dependent of Dicer processing. Nevertheless, recent findings reveal a novel pathway to generation of miRNAs depending of Drosha but in a Dicer-independent manner that relies on pre-miRNA cleavage through the endonuclease activity of AGO2 (Cheloufi, Dos Santos, Chong, & Hannon, 2010; Cifuentes et al., 2010)(Blichenberg et al., 1999; Cheloufi et al., 2010; J.-S. Yang et al., 2010). In fact, the existence of non-canonical pathways reproduces the evolutionary flexibility of miRNA biogenesis (Ha & Kim, 2014).

miRNAs biogenesis regulation

In response to developmental or environmental cues, the transcription of miRNA-encoding loci is subjected to vast changes in activity. Epigenetic mechanisms, such as DNA methylation, histone modifications and some transcription factors, namely p53, MYC, ZEB1 and ZEB2, and myoblast determination protein 1 (MYOD1) have been described as positive or negative modulators of miRNA expression (Davis-Dusenbery & Hata, 2010; A. H. Kim et al., 2010; Krol, Loedige, & Filipowicz, 2010).

Subsequently, miRNA expression can also be regulated at posttranscriptional levels by changing pri- and pre-miRNA processing (Ha & Kim, 2014; O'Carroll & Schaefer, 2012). Overexpression of monocyte chemotactic protein-1-induced protein-1 (MCPIP1) (potential miRNA-binding protein) lead to a decrease in miRNA levels whereas its knockdown had an inverse result, suggesting the existence of complex regulatory mechanism involving various RNA-binding factors that, thereby, control pre-miRNA processing and degradation (Suzuki et al., 2011).

Differences between pre-miRNA and mature miRNA expression in specific tissue have been reported, most likely due to the existence of a mechanism that regulates Dicer or AGO2 activities in a miRNA and cell type-specific manner (O'Carroll & Schaefer, 2012). Human Dicer interacts with the double-strand RNA-binding domain cofactor PACT as well as TAR RNA-binding protein (TRBP), known stabilizer of Dicer and, therefore, increasing the processing efficiency in some pre-miRNAs and mature miRNA length (Ha & Kim, 2014). Moreover, RNA binding proteins have a crucial role in modulating miRNA processing by facilitating it, like KSRP, or blocking it like LIN8 and Tut4 (Ha & Kim, 2014; O'Carroll & Schaefer, 2012; Trabucchi et al., 2009), as well as other processes such as RNA editing and methylation (Ha & Kim, 2014). Finally, neuronal activity is known to stimulate Dicer activity through the calcium-calpain system at synapse compartment, promoting the processing of miRNAs to their mature forms (Lugli, Larson, Martone, Jones, & Smalheiser, 2005).

Posttranslational modifications of RISC complex are also key regulators of mRNA repression in neurons and contribute to the processing of numerous miRNA species (O'Carroll & Schaefer, 2012). Therefore, several RISC complex components, namely AGO2, are subjected to phosphorylation that impacts negatively binding and repression of the target, while its prolyl 4-hydroxylation leads to an increase of stability or localization within P-bodies. In addition to AGO2, MOV10 can be ubiquitinated upon NMDAR-mediated activity, leading to its dissociation from the RISC and degradation mediated by proteasome pathway (Banerjee, Neveu, & Kosik, 2009; Ha & Kim, 2014). These alterations can affect the stability of RISC, resulting in its disassembly and consequent release of the repressed transcripts (Banerjee et al., 2009).

Not only miRNA stability is under regulation but also regulation of mature miRNA activity could

play an important physiological role in neurons. Therefore, based on artificial miRNA sponges developed to specifically inhibit miRNA activity in cells without changing its expression levels, it is speculated by some authors that there is a similar natural mechanism that regulates miRNA availability (Ebert, Neilson, & Sharp, 2007). Recently, it has been reported that, indeed, a class of RNAs designated as long non-coding (lnc) RNAs can regulate microRNA abundance by binding and sequestering them (Paci, Colombo, & Farina, 2014; K. Wang et al., 2014; Yue Wang et al., 2013).

miRNA targeting

After miRNA maturation, RISC is delivered into a cytoplasmic structure - the P body - comprising untranslated mRNAs (Filipowicz et al., 2008; Kosik, 2006). miRNAs recognize and bind through an imperfect base pairing to the 3'untranslated region (UTR) of target mRNA, except on the *miRNA seed sequence*, a highly conserved sequence that is localized between positions 2–8 of the 5' end of miRNA, where the biding is perfectly complementary. The seed domain is responsible for the specificity of miRNA targeting. Taking advantage of these features, bioinformatics tools have been designed allowing the prediction of putative targets of miRNAs or, inversely, the prediction of miRNAs that regulate several mRNA by the analysis of complementarity between 3'UTR and seed sequence, *in silico* and *in vitro* (Z. Hu et al., 2015; Sha, Wu, Zhang, & Guo, 2014; Siegert et al., 2015a; P. Zhou et al., 2013). On the contrary, the match of the 3' end of miRNAs with their target can be more variable. Furthermore, the downstream nucleotides, in particularly nucleotide 8 and nucleotides 13–16, although less important, also have been reported to participate in base pairing with the targets (Ha & Kim, 2014; Kosik, 2006).

In turn, dependent on the degree of complementarity between the 3'UTR and miRNA sequence, the mRNA molecule undergoes cleavage or its translation is repressed. According to a prevailing model, protein synthesis is inhibited by imperfect complementarity binding to 3'UTR of target mRNA, whereas high levels of complementarity promote cleavage of the target mRNA (Bartel et al., 2004; Filipowicz et al., 2008; Sim, Bakes, & Kaang, 2014). Although the 3'UTR seems to be the most commonly targeted sequence, some studies have reported that some miRNAs can target the 5'UTR or the coding regions of transcripts. In these cases, protein synthesis could be even enhanced instead of the usual blockade (Orom, Nielsen, & Lund, 2008; Zeng et al., 2014). Definitely, miRNA ability to suppress gene expression using multiple mechanisms increases the regulatory capacity of miRNA system.

miRNA localization

Almost 50% of all identified miRNAs are expressed in the mammalian brain. Moreover, there is a distinct pattern of miRNAs distribution and expression levels between brain areas and different types of neurons. Similarly, neurons also exhibit miRNA compartmentalization, suggesting the unique potential role of miRNAs in the regulation of local protein synthesis (O'Carroll & Schaefer, 2012). In fact, taking into account the neuron-specific diversity of miRNA expression levels, the functional role of miRNAs could be distinguished and established.

Since miRNAs are thought to operate locally to control synapse development and plasticity, it has been demonstrated, through *in situ* hybridization and synaptoneurosomes analysis, that some miRNAs are localized at dendrites (Cohen et al., 2011; G. M. Schratt et al., 2006; Siegel et al., 2009). Furthermore, proteins associated with miRNA regulation, namely Dicer, Argonaute, FMR1 and various P-body components, were found in dendritic compartment of mature neurons in a granular distribution (John Kim et al., 2004; Lugli et al., 2005). Hence, the synapse localization and activity-regulation make miRNAs a key modulator of adaptive processes of neural circuit formation and function.

Moreover, the discovery of dendritic miRNAs prompted the question of how they are transported to the dendrite. One theory defends that transport occurs after the miRNAs bind in the cell body compartment to the 3'UTR of their target mRNA, which contains the *cis* acting signal (to direct mRNA to dendrites), and hitchhike their way to the dendrites (Figure 9) (Blichenberg et al., 1999; Böckers et al., 2004; Glanzer & Eberwine, 2003; Mayford, Baranes, Podsypanina, & Kandel, 1996). Consequently, the binding of miRNAs to the 3'UTR sequence in the *cis* acting targeting signal may also prevent the dendritic translocation of mRNAs (Kosik, 2006). Likewise, miRNA can also reach dendrites by associating with FMRP RNA-binding protein (Kosik, 2006).

Alternatively, after being exported to cytosol, pre-miRNAs are assembled in RISC and Dicer complexes, which can also be transported along the dendrite and then processed locally, in an activity-dependent manner (Ashraf, McLoon, Sclarsic, & Kunes, 2006; Barbee et al., 2006; Hengst, Cox, Macosko, & Jaffrey, 2006). In this case, the selection of mRNAs potentially targeted by a specific miRNA only occurs among the transcripts present at dendrites (Kosik, 2006).

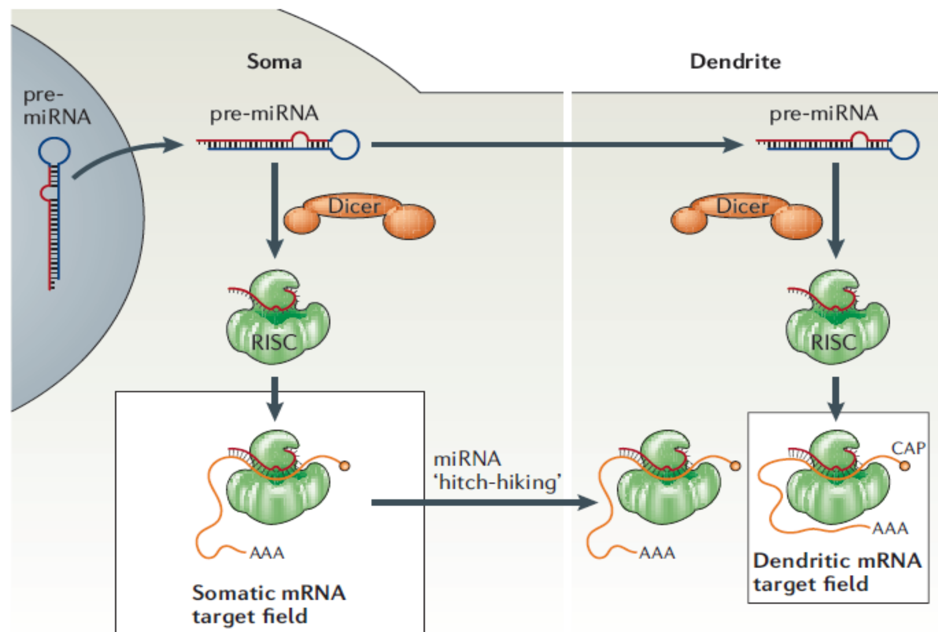

Figure 9| Models of microRNA translocation to the dendrite. Mature miRNA could travel to the dendrite bound to its target or alternatively, pre-miRNAs assembled with RISC and Dicer complexes might be transported along the dendrite and then processed and linked to their targets locally in the dendrite. (Kosik 2006)

miRNA degradation

In comparison to miRNAs transcription and biogenesis, little is known about their overall half-life in cells following processing and the mechanisms involved in their degradation. miRNAs are highly stable because both ends are protected by AGO proteins when assembled with RISC. Therefore, for miRNA decay to occur, it is likely that miRNA may need to be unloaded first, to provide access to the exonucleases. Notably, miRNA turnover in neurons appears to be highly dynamic and controlled by neuronal activity (Ha & Kim, 2014). A study by Filipowicz group demonstrated that, in retinal neurons, miRNAs have a rapid turnover in response to light adaptation; these observations were further extended to non-retinal neurons, concluding that the rapid turnover is a general property of neurons (Krol, Loedige, et al., 2010). Moreover, based on the fact that miRNAs turnover is variable, it was suggested that miRNA persistence could be related with biological functions in distinct populations of neurons (Gantier et al., 2011). And although the specific mechanisms involved in miRNA turnover, as well as what controls the specificity in their turnover remain largely unclear, theories have arisen that post-translational modifications of the proteins composing RISC may promote destabilization and RNA release (Schratt 2009 Nat neuro). So far, only MOV10, a RISC protein, was reported to be degraded through proteasome pathway, releasing translational silenced mRNAs, in a neuronal activity-dependent manner (Banerjee et al., 2009).

Regarding direct degradation of miRNAs, little is known about the signalling pathways associated with this process. Exoribonucleases XRN1 and XRN2 were found to be involved in miRNA

degradation, since their depletion raises miRNA levels (Chatterjee & Grosshans, 2009; Z. Zhang, Qin, Brewer, & Jing, 2012), but this process requires the release of the miRNA from AGO proteins, in order to provide direct access to the enzyme (Chatterjee, Fasler, Büssing, & Großhans, 2011). Studies in mammalian cells postulate pri-miRNA degradation by XRN2 following Drosha processing (Ballarino et al., 2009; Morlando et al., 2008), an effect that is diminished in the presence of miRNA target (Chatterjee et al., 2011; Chatterjee & Grosshans, 2009). Furthermore, miRNA cleavage by XRN2 may be restricted to mature neurons, since this exoribonuclease levels are higher in the nucleus of mature neurons than in immature neurons (Kinjo et al., 2013). However mechanism behind this remain to clarify, direct competition between the target and XRN2 for miRNA binding is one possible hypothesis (Libri, Miesen, Van Rij, & Buck, 2013).

Additionally, successive studies in *Arabidopsis* identified more enzymes that catalyse the breakdown of phosphodiester bonds in miRNAs (Ramachandran & Chen, 2008; Z. Zhang et al., 2012). Among them, small RNA degrading nuclease 1 (SDN1) was described to destroyed miRNAs with high efficiency in a sequence-independent manner; contrary to XRN2, SDN1 cleaves nucleotides 3' to 5' (Ramachandran & Chen, 2008). With high specificity for a small subset of miRNAs, PNPT1 and RRP41 are also required for miRNA degradation (Z. Zhang et al., 2012).

Nevertheless, miRNA degradation in neurons is still largely unknown, since the majority of studies have been performed in other organisms and/or systems, such as the data report about actions of XRN2 and XRN1v in *C. elegans*, the study showing a preferential degradation of a specific pool of miRNAs in response to interferon by PNPT1, or the study on how RRP41 and XRN1 are likely to degrade miRNAs in 293T cells (Z. Zhang et al., 2012).

miRNA biological function: from developing to mature neuron

Neurodevelopment requires coordinated expression of thousands of genes, exquisitely regulated in both spatial and temporal dimensions to achieve proper cell differentiation, localization and, furthermore, correct circuits' connectivity. miRNAs can modulate cellular differentiation and migration by regulating a wide collection of target mRNAs and growing evidence supports the idea that the miRNAs system is fundamental in all stages of neuronal development, ranging from the initial specification of neuronal cell types to the formation and plasticity of synaptic connections between individual neurons (Fiore, Siegel, & Schratt, 2008; Kosik, 2006; Petri, Malmevik, Fasching, Åkerblom, & Jakobsson, 2014a; Siegel et al., 2009; Ziats & Rennert, 2014a).

To achieve a comprehensive knowledge of miRNAs functions and dynamics throughout brain development, several studies have focused on conditional knockout (KO) or knockdown (KD) of core components in miRNA biogenesis pathway in order to understand the global role of miRNAs. The generation of KO for single miRNAs or miRNA-clusters was also performed as an approach to determine specific functional roles of different miRNAs during development in different brain structures (Petri, Malmevik, Fasching, Åkerblom, & Jakobsson, 2014b; Ziats & Rennert, 2014b). Thus, based on KO studies of Dicer, Drosha and DGCR8, miRNAs have been proposed to regulate cellular functions such as differentiation, proliferation and fate-determination of neural progenitors (Petri et al., 2014b). In fact, germline Drosha deficiency causes embryonic lethality (Cheloufi et al., 2010), and homozygous deletion of Dicer has also resulted in embryonic lethality (Petri et al., 2014a). Furthermore, cell type-specific conditional KO of Dicer results in progressive apoptotic loss of dopaminergic neurons (A. H. Kim et al., 2010), death of Purkinje cell type (Schaefer et al., 2007); likewise a selective Dicer ablation in excitatory forebrain neurons causes a reduction in brain size due to postnatal apoptosis (Davis et al., 2008; Petri et al., 2014b). Taken together, these data strongly confirm the requirement and function of miRNAs in brain development. To note, results of disrupted miRNA biogenesis could be masked to unknown extent by the disturbance of other cellular functions independent of miRNAs but fulfilled by the same enzymes. Likewise, the ability of an organism or cell to bypass a single gene KO or KD is an inherent problem that should be taken into account (Cheloufi et al., 2010; Kosik, 2006; Yangming Wang, Medvid, Melton, Jaenisch, & Blelloch, 2007).

Therefore, it becomes primordial to understand the function of a specific miRNAs during brain development, assessing it through individual miRNA KO approaches. However, this methodology entails several difficulties. In the first place, one miRNA can be transcribed from multiple loci throughout the genome and within other genes, making KO extremely challenging (Petri et al.,

2014b). Important to consider, regarding miRNA redundancy as a result of many miRNAs sharing the same seed sequence, single KO approach could converge into a phenotype with no apparent alterations, since the ablation of one miRNA is compensated for by another one. For example, miRNA-449 cluster, which encodes miRNA-449a, miRNA-449b, and miRNA-449c; miRNA-34b/c cluster, encoding miRNA-34b and miRNA-34c; and miRNA-34a have the same seed sequence. Consequently, single KO mice of miRNA-34a, miRNA-34b/c, or miRNA-449 exhibit no discernible phenotype (Bao et al., 2012; Wu et al., 2014). Similarly, because miRNAs are often transcribed in clusters, deletion of only one miRNA gene could, therefore, disturb the expression of another miRNA embedded in the cluster. Despite these difficulties, several studies have confirmed the role of specific miRNAs in different stages and processes of neurodevelopmental as it will be discussed further. This overview will not be an extensive review of these topics, but will focus its attention in specific miRNAs as examples of their role in a determined process or mechanism.

Neuronal patterning

It is believed that, during embryonic development, altered expression of miRNAs modulates the neuronal patterning of anterior-posterior or dorsal-ventral axes, which are under the Homeobox (Hox) gene control. In fact, findings in Zebrafish demonstrate that miRNA expression profile in later stages is diverse, whereas during early development miRNAs seem to be generally absence (Wienholds et al., 2005). Conversely, expression of *Drosophila* miRNAs, at the onset of zygotic transcription in early embryos, displays a diverse and dynamic pattern. Indeed, great variety in the spatial arrangements of miRNA transcription is observed, and some miRNAs are activated in specific germ layers or present in particular organs or differentiating cells (Aboobaker, Tomancak, Patel, Rubin, & Lai, 2005). In this study, miRNAs from the miRNA-309/3/286/4/5/6 cluster are initially expressed broadly, but repressed later and continue to spatially change rapidly during posterior stages. Coupled with this cluster, vertebrate miRNA-10 expression is also modulated along the embryonic anterior-posterior axis, targeting the neighbouring Hox gene mRNAs, since its sequence is located between the Hox4 and Hox5 orthologues. In addition to miRNA-10, genes of miRNA-196 miRNA family are embedded in vertebrate Hox clusters, encoding miRNA-196a1, miRNA-196a2 and miRNA-196b (Mansfield et al., 2004). Moreover, both in mouse embryos and in cell cultures, miRNA-196 was observed to repress HoxB8 mRNAs through its cleavage, given the perfect complementarity between miRNA-196 and the Hoxb8 3'UTR. Although Hoxb8 coordinates more anterior axial regions, paralogous group 8 Hox genes in mouse are involved in patterning an extensive anterior–posterior axial region (Kosik, 2006). Hence, restriction of miRNA-196a in posterior region is predicted to rule in way of proper embryonic patterning (Kosik, 2006;

Mansfield et al., 2004).

Although most miRNAs maintain a fairly constant level of expression when neurons are differentiating, a small number of miRNAs has been reported to markedly alter their levels during development. miRNA-92a is an example since it presents high expression in neuronal progenitors but is nearly absent in differentiated neurons, whereas miRNA-124 and miRNA-29a present the inverse pattern of expression (Fiore et al., 2008). Overall, this type of data provides the basic knowledge to further study miRNA functions.

Establishment and maintenance of cell identity

Changes in the expression level of only a few specific miRNAs could underlie specificity of individual cell types in a tissue and maintenance of cell fate identity (Fiore et al., 2008; Kosik, 2006). To illustrate this idea, in mammals, miRNA-124 and miRNA-9 are highly upregulated at the onset of embryonic neurogenesis and restricted to the central nervous system. Moreover, when overexpressed, both miRNAs shift the differentiation toward a neuronal fate, and the opposite effect is achieved when they are inhibited, due to their role in anti-neuronal pathways suppression (Siegel et al., 2009; Sim et al., 2014). Cell types go through distinct non-overlapping competency states, and miRNA-430 has been described to counteract the formation of mixed states, maintaining sharp boundaries between developmental transitions and employing a exactly switch from one state to another (Giraldez et al., 2005; Kosik, 2006). As a result, miRNAs are active throughout the transition from neural progenitor cells to neurons and astrocytes (Fiore et al., 2008; Kosik, 2006).

Axonal pathfinding

After spatial and temporal patterning of developing neurons, the building of circuitries of the nervous system takes place, in which appropriate pathfinding, guided by growth cones, is required (Kosik, 2006). Interestingly, miRNA-124-1 KO mice displayed abnormal outgrowth of axons in the dentate gyrus, highlighting the possible role of miRNAs in axonal pathfinding (Sanuki et al., 2011). Additionally, a recent work demonstrates evidence that miRNA-29c has an important role in neurite outgrowth and mice brain development via down-regulating PTEN (Phosphatase and tensin homolog) expression (Zou et al., 2015).

Apoptosis

The progressive development of the brain comprising novel structures arising is accompanied by massive loss of neurons and glia. Accordingly, growing evidence points to miRNAs having a role

in apoptosis (Kosik, 2006). Indeed, studies in Drosophila embryos demonstrate that the miRNA-2 family, comprising miRNA-2/6/11/13/308, is required for apoptosis suppression by targeting the pro-apoptotic factors. Indeed, all family members have as putative target proapoptotic factors hid, grim, reaper and sickle (Leaman et al., 2005). Furthermore, via an indirect target, miRNA-146a mediates induction of proapoptotic caspase-3, thereby increasing cell apoptosis, in upport of the idea that miRNAs' machinery has a role on regulating the cell-cycle program (B. Zhang et al., 2016).

Dendritic growth and arborisation

An important feature in neurons is the colossal number of contacts that each neuron forms with other neurons, via axons and dendrites. For this reason dendrites are considered the receiving stations for incoming synaptic inputs and the architecture of the dendritic arbor determines how many synapses will form and how the transmitted information by these synapses is integrated (Kasai, Matsuzaki, Noguchi, Yasumatsu, & Nakahara, 2003; Kosik, 2006; G. Schratt, 2009). Therefore, dendrite growth and remodelling are processes highly regulated and the knowledge of their molecular mechanisms is extremely important to understand the development of neural circuits. Consequently, the contribution of de novo protein synthesis for these dynamic processes is recognized to be regulated by miRNAs (G. M. Schratt et al., 2006; Siegel et al., 2009).

Accordingly, miRNA-132 seems to mediate dendritic growth, since, either in newborn neuronal cultures or *in vivo* mice models, this miRNA is both necessary and sufficient for the growth of neurites that later become dendrites (Magill et al., 2010). In the same way, besides the role in the establishment of neuron fate, miRNA-124 shows growth-promoting function. Hence, shared mechanism for induction of dendritic growth, by both miRNAs, relies on downregulation of small Rho GTPases, key regulators of actin cytoskeleton at dendritic compartment (Magill et al., 2010; G. Schratt, 2009). Subsequent studies using more mature cultures noticed that miRNA-132 not only induces dendritic-growth mediated through neuronal activity but also spine formation (Impey et al., 2010; Wayman et al., 2008). On the contrary, miRNA-134 negatively regulates dendritic remodelling in an activity-dependent manner, by repressing the translation of Pumilio-2 (PUM2) mRNA. Taken together, the regulation of gene expression in response to neuronal activity works to shape dendritic arbors (G. Schratt, 2009).

Further data provided by genetic studies supports the notion that miRNA system regulates dendritic arborisation. In studies performed in *Drosophila*, overexpression of RNA helicase Me31B, FMR1, PUM, proteins involved in miRNA regulation, lead to altered branching of terminal dendrites in sensory neurons (Barbee et al., 2006). Additionally, FMR1 affected steady-state levels of miRNA-124, corroborating its role in regulation of miRNA biogenesis, but extending its function to miRNA

activity (G. Schratt, 2009).

Spine formation and maturation

From dendritic shaft small protrusions arise to form dendritic spines, where most of the excitatory synapses are received. Dendritic spines present a plastic structure, both during neuronal activity or even at rest (Kasai et al., 2003; Rochefort & Konnerth, 2012). Thus, determined by cytoskeleton, spines can display large heads – stubby and mushroom – stable structures, expressing a large number of AMPARs, and which contribute to strong synaptic connections, whereas small head spines – filopodia and thin – change their shape rapidly, underlying weak and silent synapses in learning and adaptive processes (Kasai et al., 2003; Rochefort & Konnerth, 2012). As a consequence of the variety of shape and size, high degree of functional diversity suggests that large and small spines are “memory” spines and “learning” spines, respectively (Kasai et al., 2003).

In fact, miRNA pathways seem to have an important involvement in synapse growth and maturation, although miRNA are dispensable for the initial formation of synaptic contacts, because only alterations in morphology and not in spine number were found in Dicer-null mice neurons (Davis et al., 2008). Moreover, in neocortical pyramidal neurons of FMRP-null mice, the development of dendritic spines fails to proceed normally, resulting in longer, phenotypically immature spines (Galvez & Greenough, 2005). Moreover, findings suggest that miRNA-134, besides regulating dendrite arborisation, may perturb the morphology of pre-existing spines in a mechanism dependent on Lim-domain-containing protein kinase 1 (Limk1). Limk1, a positive regulator of actin filaments polymerization, is repressed by miRNA-134 in the absence of synaptic plasticity. However, under synaptic stimulation with BDNF, the blockage of translation is relieved due to miRNA inhibition, allowing Limk1 translation, and therefore enhancement in spine growth (G. M. Schratt et al., 2006; Sim et al., 2014). Nevertheless, it is a challenge to comprehend the contribution of local translational control in dendrites to plasticity, due to the presence of locally synthesized proteins and those transported from the cell body (Kosik, 2006). Analogous to miRNA-134, miRNA-138, a brain-enriched miRNA localized within dendrites, decreases the size of dendritic spines without altering the total number of synapses (Siegel et al., 2009). Indeed, miRNA-138 inhibition using 2’O-Me AS oligonucleotides results in a robust and significant spine volume increase. This miRNA mediates its effects by increasing $G\alpha_{13}$ palmitoylation and membrane localization, resulting in Rho activity raise that triggers spine shrinkage (Siegel et al., 2009).

Overall, given the crucial role of spine actin cytoskeleton in synaptic plasticity, the novel miRNA-dependent layer of regulation of actin signalling pathway is an important player in adaptations in individual spines as a response to synaptic activity (G. Schratt, 2009; Siegel et al., 2009).

Neuronal activity-dependent regulation of microRNAs

At synapses, local translation of mRNAs is repressed until neurons receive appropriate extracellular stimulus, and upon LTP or LTD stimulus, protein synthesis takes place in order to allow long-lasting alteration in synaptic strength. For this reason, these complex processes involving coordinated regulation of gene networks require fine-tune by a tight temporal and special regulated system – miRNAs (Joilin et al., 2014; B. Ryan et al., 2015a; Sim et al., 2014). Thus, miRNAs levels can be rapidly regulated in response to external stimuli. In turn, their rapid regulation plays a crucial role in synaptic plasticity by either promoting or inhibiting local translation, which will adjust functionality and structure of dendritic spines. *Drosophila* studies have shown that diminished levels of Armitage at synapses coincide with increase of synaptic protein synthesis that underlies stabilization of memory. Therefore, upon synaptic activation, control of RISC pathway is required for long-lasting memory to overcome block-mediated synthesis by miRNA pathway (Ashraf et al., 2006). Accordingly, screening studies in several neuronal models have noticed a pool of activity-regulated miRNAs that respond to different neuronal activity manipulation paradigms, like LTP, LTD and homeostatic plasticity (Eacker, Keuss, Berezikov, Dawson, & Dawson, 2011; Kye et al., 2011; Mellios et al., 2011; Pai et al., 2014a; C. S. Park & Tang, 2009; Sim et al., 2014; Wibrand et al., 2010).

LTP

As a response to synaptic plasticity and memory formation, the miRNA system controls post-transcriptional regulation in a neuronal activity-dependent manner selectively at individual synapses (Figure 10) (Nikkie F.M. Olde Loohuis et al., 2015; Sim et al., 2014). For instance, miRNA-134-5p inhibits translation of Limk1 at the synapse until synaptic activation occurs. When stimulated with BDNF, degradation of miRNA-134-5p leads to Limk1 expression and therefore to spine growth (G. M. Schratt et al., 2006; Sim et al., 2014).

Conversely, it is interesting to realize that synaptic activation could also activate miRNAs, potentially blocking protein synthesis of negative regulators of LTP mechanisms. Thus, upon 15 min of chemical-LTP (cLTP), hippocampal slices microarray revealed an increase of the majority of miRNAs evaluated in the screen (C. S. Park & Tang, 2009). Moreover, *in vivo* studies, where electroconvulsive shocks were applied in order to generate massive depolarization, reported an increase in the screened miRNAs within 1 h (Eacker et al., 2011). Importantly, a recent work in *in vivo* awake rat model using Affymetrix miRNA arrays noticed upon 20 min of HFS-induced LTP, the majority of mature miRNA transcripts analysed were downregulated, including miRNA-132-

3p and miRNA-34a-5p that was reported by Wibrand and colleagues to be induced (Joilin et al., 2014; Wibrand et al., 2010). Nevertheless, discrepancies of obtained results may be explained due to different stimuli protocols used or different RNA extraction methods. Similarly, activation of *in vitro*-cultured mouse neurons by the neurotransmitter glutamate induced a reduction in the levels of several miRNAs including miRNA-124, miRNA- 128, miRNA-134, and miRNA-138, although no changes on miRNA-16, miRNA-25, miRNA-23b, and miRNA-132 were verified, pointing to a different response to neuronal activation by different groups of miRNAs (Krol, Busskamp, et al., 2010). Hence, as shown by Pai and colleagues, upon HFS-induced LTP, miRNA levels are modulated throughout time, possibly contributing to a fine-tune of each molecular change underlying LTP (Pai et al., 2014a). Therefore, when miRNA expression was analysed at later time points, it was observed that the increase of miRNA levels was transient, followed by a global decline and, 24 h after stimuli, miRNA levels returned to basal levels (Eacker et al., 2011). Together, these results support the idea that reduction of miRNAs levels correlates with increased amount of protein synthesis during L-LTP phase, by releasing the target mRNA from translation suppression (Joilin et al., 2014; B. Ryan et al., 2015a; M. M. Ryan et al., 2012).

Moreover, observed changes of miRNA levels could be due to related forms of activity-dependent synaptic simulation. For instance, miRNA-501-3p inhibits GluA1 expression in a NMDAR-dependent manner, since increased miRNA-501-3p expression is blocked by the NMDAR antagonist AP-V (Z. Hu et al., 2015). Differently, induction of miRNA-132, regulated by CREB, requires not only the activation of NMDARs but also two additional pathways: CaM K and MEK-ERK (Cheng et al., 2007; Sim et al., 2014; Wayman et al., 2008). Several miRNAs are positively enhanced in response to several neuronal activity paradigms, such as BDNF, KCl, bicuculline, seizure, contextual fear conditioning, odorant stimulus, light, cocaine intake and visual stimulus in particular brain regions of living animals (Sim et al., 2014). In addition, miRNA-124 has been studied largely for its role in neuronal development but a study in *Aplysia californica* demonstrates its activity-dependent regulation. Thus, inducing long-term facilitation at sensory-to-motor synapse, by applying five spaced pulses of 5-hydroxytryptamine (5-HT), led to a decrease of mature miRNA-124 through the MAPK signalling pathway (Martin et al., 1997; Rajasethupathy et al., 2009).

However, the total levels of miRNAs in the cell does not represent the total amount of mature miRNA loaded into RISC and, consequently, ready to target mRNAs. Whether neuronal activity affects miRNAs functionality remained unclear. Therefore, the total amount of miRNA bound to AGO2 after neuronal activity stimulation was measured and it was reported that ratio of AGO2/total miRNA expression was modulated in specific miRNAs. In fact, variations in miRNAs levels, as well as their association with AGO2, was specific to NMDA receptor-dependent LTP induction, since changes were reversed when HFS was applied in the presence of NMDAR antagonist (Pai et

al., 2014b).

Although findings point to modulation of miRNAs in response to LTP induction, it is important to understand that does not prove their contribution in LTP mechanisms (B. Ryan et al., 2015a). Nevertheless, it is believed that some miRNAs could influence the maintenance of LTP by tuning different related mechanisms of LTP maintenance. One study has demonstrated that miRNAs regulate neurotransmitter release, ending up altering synaptic transmission and strength. miRNA-25-3 and miRNA-185-5p have sarco/endoplasmic reticulum calcium ATPase (SERCA2) as target, which maintains within a range of amount of calcium in endoplasmic reticulum (Earls et al., 2012). As a result, in cases of SERCA2 overexpression and concomitant decrease expression of these miRNAs, the outcome is a raise of presynaptic terminal calcium, thereby, increasing neurotransmitter release and enhancement of LTP (Earls et al., 2012). Additionally, a recent study demonstrated that gain of function of miRNA-137, a miRNA related to schizophrenia, reduced vesicle release in stimulated neurons, resulting in reduced LTP induction as well as impairment in hippocampus-dependent learning (Siegert et al., 2015b).

Besides neurotransmitter release facilitation, trafficking and surface abundance of AMPARs are key mechanisms that drives Hebbian plasticity in the postsynaptic compartment of mature synapses (Bassani et al., 2013; Ju et al., 2004; B. Ryan et al., 2015a). Hence, miRNAs contribute to postsynaptic effects of synaptic plasticity through direct and indirect regulation of glutamate receptor subunits trafficking. Moreover, subunit composition of glutamate receptors that contribute to the sustained structural and functional changes underlying long-term plasticity seems to be modulated by the miRNA system. For example, among of a pool of miRNAs that alter synaptic strength, miRNA-138 and mRNA-181 are related to decreased GluA2-containing AMPAR cluster size and consequently the amplitude and frequency of postsynaptic currents (Cohen et al., 2011; Saba et al., 2012; Siegel et al., 2009). On the other hand, upon BDNF stimulation, increased miRNA-132 levels seem to induce marked upregulation of glutamate receptors, not only GluA1-containing AMPARs, but also GluN2A and GluN2B subunits of NMDARs, suggesting that miRNA-132 has a positive effect on the upregulation of postsynaptic proteins mediated by BDNF (Kawashima et al., 2010). Furthermore, miRNA-125b was found to target the GluN2A subunit of NMDARs, prolonging NMDAR-EPSCs in consistence to reduction of GluN2A expression (Edbauer et al., 2010). Besides the direct regulation of receptor number, alterations in excitatory synaptic function could be mediated by alterations on the signalling pathway. For example, miRNA-219 negatively regulates the function of NMDARs, by targeting CaMKIIγ (Kocerha et al., 2009).

Therefore these studies demonstrate that different miRNAs contribute to activity-dependent local synthesis of AMPARs as well as NMDARs in dendrites (Z. Hu et al., 2015).

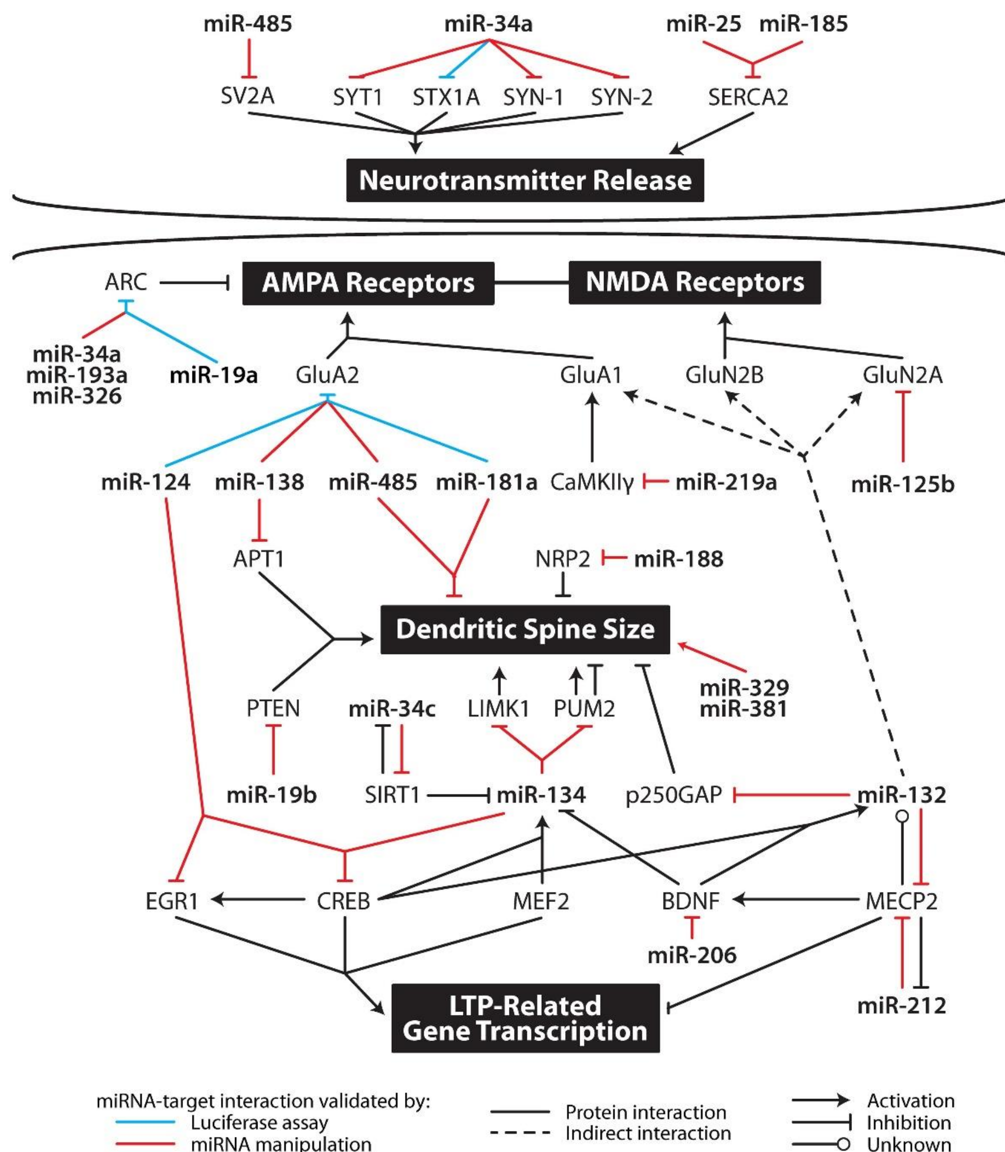

Figure 10| Multi-level contribution of microRNA to synaptic plasticity. MicroRNA likely influence the translation of multiple mRNA important in specific aspects of synaptic plasticity including neurotransmitter release, AMPA and NMDA receptor subunit levels, dendritic spine size, and gene transcription. APT1, acyl-protein thioesterase 1; ARC, activity-related cytoskeleton-associated protein; BDNF, brain-derived neurotrophic factor; CaMKIIγ, calcium/calmodulin-dependent protein kinase II gamma; CREB, cAMP response element-binding protein; EGR1, early growth response 1; GluA1, glutamate receptor, ionotropic, AMPA 1; GluA2, glutamate receptor, ionotropic, AMPA 2; GluN2A, glutamate receptor, ionotropic, NMDA 2A; GluN2B, glutamate receptor, ionotropic, NMDA 2B; LIMK1, LIM domain kinase 1; MECP2, methyl CpG binding protein 2; MEF2, myocyte enhancer factor-2; NRP2, neurophilin 2; PTEN, phosphatase and tensin homolog; PUM2, pumilio homolog 2; SERCA, sArco/endoplasmic reticulum Ca2+ ATPase; SIRT1, sirtuin 1; STX1A, syntaxin 1a; SV2A, synaptic vesicle glycoprotein 2A; SYN1, synapsin I; SYN2, synapsin II; SYT1, synaptotagmin I. (B. Ryan, Joilin, & Williams, 2015b)

LTD

Comparing to LTP, little is known about miRNA involvement in LTD mechanisms. So far, miRNA studies performed in hippocampal slices revealed an increase of most of the miRNAs screened, upon 30 minutes of mGluR-LTD (C. S. Park & Tang, 2009). To note, synaptic plasticity on postsynaptic compartment is also regulated by miRNA-137, since this miRNA represses the

incorporation of AMPARs into the synaptic membrane, preventing plasticity (Nikkie F.M. Olde Loohuis et al., 2015). Thus, the authors suggest that this miRNA triggers a selective removal of GluA1 from the synapse by targeting directly actin cytoskeleton dynamics. Taken together, this work provides evidences that miRNA-137 is necessary for mGluR-LTD (Nikkie F.M. Olde Loohuis et al., 2015). Similarly miRNA-125a targets PSD95 and promotes a decay on PSD95 mRNA translation,; this inhibitory effect is required for the mGluR-mediated regulation of PSD95 translation in neurons (Muddashetty et al., 2011). Furthermore, evidence points to upregulation of miRNA-135 to mediate suppression of complexin-1/2 expression, found to stabilize spine shrinkage in LTD due to AMPARs exocytosis inhibition (Zhonghua Hu et al., 2014).

However, deep sequencing to profile the miRNA transcriptome upon NMDARs-dependent LTD induction in the mouse hippocampus, complemented with live imaging and electrophysiology studies, identified miRNA-191 downregulation to be essential for sustained spine remodelling and induction of synaptic depression in LTD (Zhonghua Hu et al., 2014). Decreased expression of this miRNA causes accumulation of its target, tropomodulin 2 (Tmod2), which is required for spine remodelling during LTD (Zhonghua Hu et al., 2014). Decreased miRNAs expression fits with the protein synthesis-requirement of LTD. Conversely, miRNAs upregulation reveals unexpectedly a new dimension of proteome regulation in LTD, in other words, a mechanism likely to be regulated by translational inhibition (Zhonghua Hu et al., 2014).

Homeostatic plasticity

In studies of homeostatic plasticity, chronic activity blockade with voltage-gated sodium channel blocker TTX or chronically increase neuronal activity blocking $GABA_A$ receptor with bicuculline generate prolonged changes on synaptic activity in neuronal cultures and, consequently, altered the expression of miRNAs (van Spronsen et al., 2013). Notably, upon chronic increase of activity with bicuculline/4-aminopyridine (4-AP), hippocampal neurons exhibit an increase of both the precursor and mature forms of miRNA-485. miRNA-485, targets synaptic vesicle protein SV2A, regulates neurotransmitter release, and promotes a decrease in spine density and maturation, PSD95 clustering and GluA2 surface expression (Cohen et al., 2011). In addition to modulation of miRNA-485 levels in response to chronic increase of activity, miRNA-134 was also found upregulated, specifically at the synapto-dendritic compartment. Moreover, miRNA-134 is required for early synapse elimination and late structural rearrangements of excitatory synapses on scaling down, due to the targeting of RNA-binding protein PUM2 in response to miRNA-134 increase. These finding show miRNA-134-dependent elimination of specific synapses and removal of GluA2-containing AMPA receptors from the surface of the neuronal membrane (Fiore et al., 2014).

Inversely, during neuronal activity blockade, the inhibition of GluA1 mRNA translation mediated

by the miRNA-92a is released, resulting in incorporation of new GluA1 receptors at synapse and consequently increase in AMPAR-mEPSC amplitudes. miRNA-92a overexpression blocks synaptic scaling up, prompting to the conclusion that homeostatic scaling requires the miRNA-92a–dependent de-repression of GluA1 translation (Letellier et al., 2014).

Emerging data now suggest a role for the miRNA-29a/b cluster as a fine-tuning regulator of synaptic strength and neuronal plasticity, primarily on postsynaptic spine structure. Hence, in conditions of enhanced neuronal activity, through targeting a regulatory subunit of Arp2/3 complex – Arpc3, upregulated miRNA-29a/ cluster regulates hippocampal spine remodeling, and the same role is suggested during contextual fear learning (Lippi et al., 2011).

Mechanisms behind neuronal-activity miRNA regulation

As discussed so far, miRNA biogenesis and processing is a highly regulated cascade of events. Hence, in neurons, it is possible that neuronal activity can influence each step (Figure 11) (Lugli et al., 2005; G. Schratt, 2009; Sim et al., 2014).

miRNA gene transcription is the first layer of miRNA control, where the increase in intracellular calcium levels triggers downstream pathways that stimulate activity-regulated transcription factors, such as CREB and myocyte enhancer factor-2 (Mef2) (Fiore et al., 2009; Nudelman et al., 2010; Remenyi et al., 2013; G. Schratt, 2009; Vo et al., 2006; Wayman et al., 2008). For instance, it is known that the transcription of miRNA-132 and miRNA-212, which reside in the same gene locus, is regulated via CREB-dependent mechanisms upon high neuronal activity (Magill et al., 2010; Nudelman et al., 2010; G. M. Schratt et al., 2006; G. Schratt, 2009; Sim et al., 2014; Wayman et al., 2008). Also, miRNA-379-410 cluster transcription is triggered by neuronal activity, via Mef2 transcription factor, and the dendritic miRNA-134, a known regulator of dendritic arborisation and spine morphogenesis, is part of this cluster and (Fiore et al., 2009).

Another highly regulated step is Dicer activation. It has been proposed that under acute neuronal stimulation of excitatory synapses, increased calcium influx activates calpain. This enzyme has the capacity to cleave inactive Dicer enriched at PSD, giving rise to an active 75 KDa fragment, enhancing Dicer RNA III activity (Lugli et al., 2005; Sim et al., 2014). Equally important, a mechanism relying on evidence that pre-miRNA processing could be prevented by RNA-binding proteins (RBPs), has been suggest. Thereby, under low neuronal activity, binding of RBPs to miRNA blocks Dicer-mediated processing, which is released upon high activity levels allowing Dicer to access the pre-miRNA stem loop sequence (G. Schratt, 2009).

Also, dendritic P-body like structures containing miRNAs can respond to neuronal activation, suggesting that they may control the levels of miRNA available locally to target mRNAs. Notably,

miRNA transport offers another point of regulation dependent on activity (Cougot et al., 2008; G. Schratt, 2009). Therefore, in conditions of low activity, either mature or pre-miRNA transport is decreased, preventing accumulation of miRNA and, subsequently, downregulation of target mRNAs in dendritic compartment. High activity conditions promote dendritic miRNA transport, which is due to regulation of RNA transport granules (G. Schratt, 2009).

Although miRNAs are generally assumed to have a very long half-life, neuronal miRNAs often play a role in rapid process such as developmental transitions or synaptic plasticity (Bhattacharyya, Habermacher, Martine, Closs, & Filipowicz, 2006; Gantier et al., 2011). In fact, many neuronal miRNAs present a turnover much faster than miRNAs on other cell types, since blocking action potentials or glutamate receptors strongly affect miRNA turnover rates (Krol, Loedige, et al., 2010). This fast downregulation of miRNAs was observed during dark adaptation in retinal neurons followed by increased transcription as a consequence (Krol, Loedige, et al., 2010). Additionally, rapid reduction of miRNAs was also observed in organotypic hippocampal slices, hippocampal and cortical cultures, and even neurons derived from mouse embryonic stem cells (Krol, Loedige, et al., 2010; Sim et al., 2014). However, as referred previously, miRNA turnover and underlying pathways remain to be elucidated. Nonetheless, following neuronal activity, one of the RISC components in *Drosophila*, Armitage, is rapidly degraded by proteasome (Ashraf et al., 2006). The lack of Armitage lead to release translational suppression of synaptic mRNAs, such as CaMKII, whose translation is related with LTP maintenance (Ashraf et al., 2006). Likewise, MOV10, a mammalian ortholog of Armitage, is rapidly degraded by the proteasome in an NMDAR-mediated activity-dependent manner. This decay culminates on a release miRNA-138 mediated suppression of several neuro plasticity regulating genes including CamkII, Limk1, and Lypla1 (Banerjee et al., 2009). In the same way, RISC remodelling after synaptic stimulation is associated with posttranslational modifications of its components or attachment of RBPs, although the specific signalling pathways are unknown (G. Schratt, 2009).

Overall, miRNAs and respective associated protein complexes emerge in a complex activity-regulated signalling network, which allows them to control adaptive and dynamic processes, especially dendritic growth and remodelling or synaptic plasticity (G. Schratt, 2009).

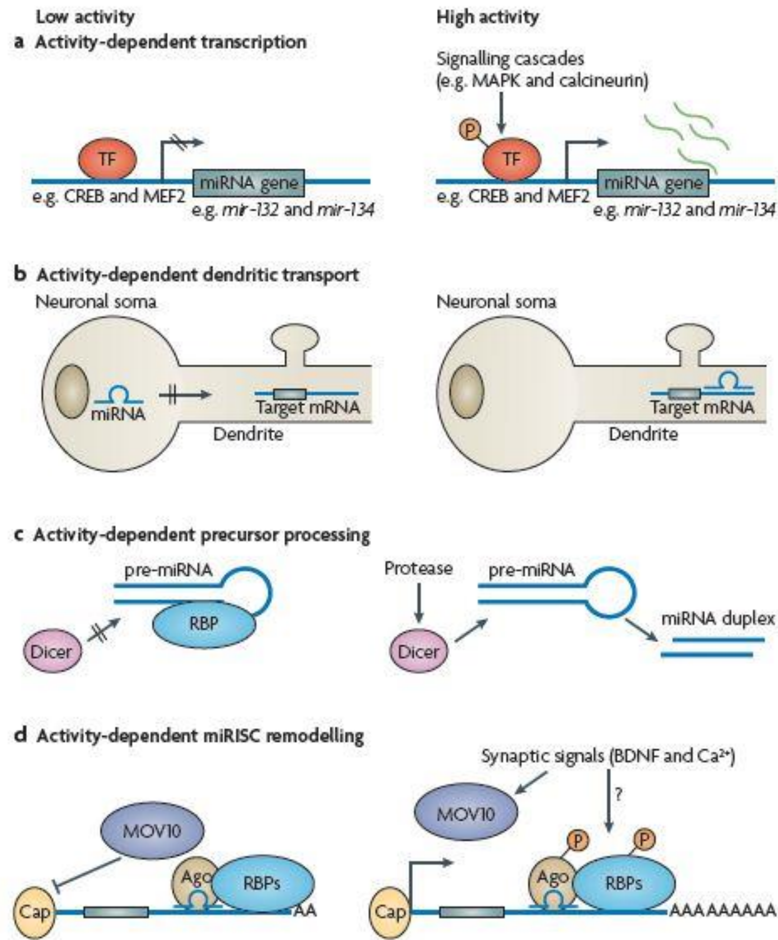

Figure 11| Neuronal activity could regulate miRNAs by different mechanisms. Several neuronal miRNAs are subject to regulation by neuronal activity at transcription level (**A**), at transport along the dendrites (**B**), at processing (**C**) by miRNAISC remodelling **(D)**. (G. Schratt, 2009)

miRNA-132 and miRNA-186 characterization

miRNA-132 and miRNA-186 in hippocampal neurons are the focus of the present work. Therefore, the next sections address what is currently known about their regulation and functions in neurons.

miRNA-132

The first and most studied miRNA induced by neuronal activity is miRNA-132. Transcribed from only a single locus – miRNA 212/132 locus – these two miRNAs share the same primary transcript but are processed into different miRNA precursors by Drosha and DGCR8 (Cheng et al., 2007; Magill et al., 2010; Sim et al., 2014). Their transcription is under regulation of rapid-response transcription factor CREB, activated during dendritic development and neuronal synaptic plasticity (Cheng et al., 2007; Vo et al., 2005). In further detail, NMDARs activation induces CaM kinase kinase (CaMKK), CaMKIγ, ERK1/2 and MEK-ERK pathways resulting in activation of its downstream target CREB (Kawashima et al., 2010; Remenyi et al., 2013; Wayman et al., 2008; Wibrand et al., 2010). Although, the majority of studies report an increase in miRNA-132 levels with high neuronal activity (Eacker et al., 2011; Pai et al., 2014a; Wibrand et al., 2010), it was also described to be decreased upon 20 minutes of HFS-LTP induction in vivo (Joilin et al., 2014). Notably, upon 20 min of LTP induction, miRNA-132 was downregulated (Joilin et al., 2014), becoming up-regulated 2 h after LTP induction (Pai et al., 2014a; Wibrand et al., 2010). To further support the idea of miRNA-132 regulation by neuronal activity predominantly via CREB, KCl treatment increased its precursor, being attenuated through CREB family inhibitor (ACREB) expression (Wayman et al., 2008).

Furthermore, the involvement of this miRNA in morphogenesis of developing neurons has been demonstrated by several groups (Edbauer et al., 2010; O'Carroll & Schaefer, 2012). Briefly, by targeting GTPase-activating protein p250GAP, miRNA-132 induces local increase of Rac and Cdc42 activity, which in turn promotes spine growth, likely through Limk1-mediated actin polymerization (Figure 12) (Magill et al., 2010; G. Schratt, 2009; Wayman et al., 2008). Besides modulating p250GAP, miRNA-132 also regulates dendritic growth by controlling methyl CpG-binding protein2 (MeCP2), p120RasGAP and p300 RasGAP levels (Magill et al., 2010; Remenyi et al., 2013; Wayman et al., 2008). The role of miRNA-132 in spine maturation was further clarified in studies where this miRNA was overexpressed, promoting formation of stable mature mushroom and stubby spines, but decreasing overall spine number; or inhibited, increasing the proportion of immature filopodia spines (Edbauer et al., 2010; Mellios et al., 2011; B. Ryan et al., 2015a).

In vitro studies show also that miRNA-132 regulates dendritic outgrowth and branching in an activity-dependent manner (Wayman et al., 2008). It is believed that, *in vivo*, induction of this miRNA might be related to critical synaptic refinements (Nudelman et al., 2010; Wayman et al., 2008). Additionally, Hancock and colleagues demonstrated that miRNA-132 is not only involved in dendritic outgrowth and remodelling but also in axon extension of developing neurons, by targeting Ras-GTPase activator Rasa 1 (Hancock, Preitner, Quan, & Flanagan, 2014). In the same way, hippocampal neurons of miRNA-132 knockout mice display abnormal dendrite length, arborization, and spine density. Together these studies show an important role for miRNA-132 in regulating axonal growth, dendritic arborisation and dendritic spine morphology (Magill et al., 2010).

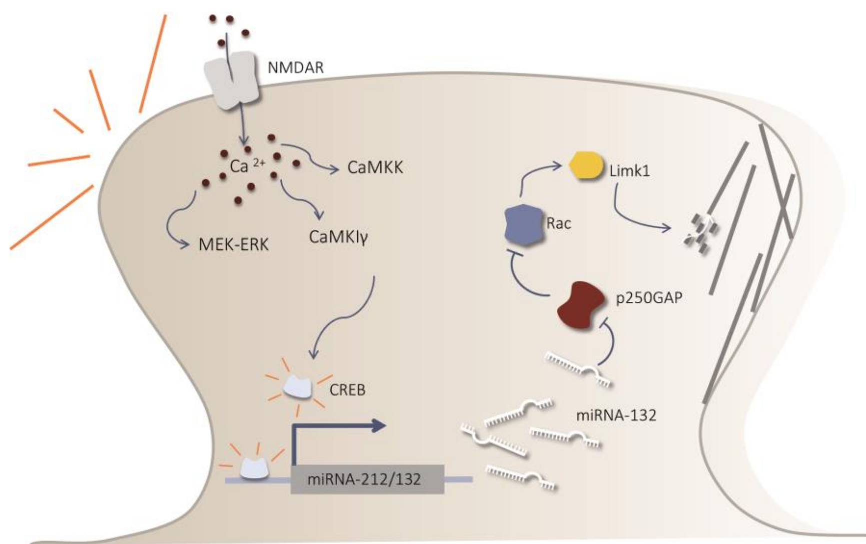

Figure 12| Model for the role of miRNA-132 in the regulation of spine morphogenesis. Activation of NMDARs enhances MEK-ERK; CaMKIIγ and CaMKK pathways. As a consequence downstream transcription factor CREB is activated promoting miRNA-132 transcription. By targeting GTPase-activating protein p250GAP, miRNA-132 induces local increase of Rac and Cdc42 activity, which in turn promotes spine growth, likely through Limk1-mediated actin polymerization.

Since alterations in spine size are accompanied by alterations in synaptic strength, studies have evaluated the role of miRNA-132 in synaptic plasticity. *In vitro* miRNA-132 overexpression increases the mean mEPSC amplitude and frequency, but inhibiting miRNA-132 did not affect either amplitude or frequency of mEPSCs. However, mice with a deletion of miR212/132 cluster show an increase in both mEPSC amplitude and frequency (Edbauer et al., 2010; Remenyi et al., 2013). Moreover, BDNF is known to promote upregulation of miRNA-132 levels (Vo 2005; Klein, 2007; Hawashima, 2010; Ryan, Joilin, & Williams, 2015b), through the TrkB and ERK1/2 pathways (B. Ryan et al., 2015b). This effect is directly correlated with upregulation of GluN2A, GluN2B and GluA1, suggesting that miRNA-132 is involved in BDNF-mediated upregulation of postsynaptic proteins (Kawashima et al., 2010). Moreover, miRNA-132 was also reported to be part

of the homeostatic regulation of BDNF levels by inhibiting MeCP2, an important regulator of BDNF transcription (Klein et al., 2007).

The increase on AMPARs number explains the observed difference in amplitude of synaptic currents (Remenyi et al., 2013). Thus, miRNA-132 seems to be involved in regulation of pre- and postsynaptic protein expression, playing a role on NMDA-dependent neuronal plasticity (Edbauer et al., 2010; Wayman et al., 2008). Furthermore, MeCP2-mediated miRNA activity controls excitatory synaptic strength by regulating glutamatergic synapse number in hippocampal neurons (Kawashima et al., 2010). Some studies point a reciprocal inhibition, *i.e.*, MeCP2 inhibits miRNA-132 *in vitro* whereas in *in vivo* conditions, miRNA-132 inhibits MeCP2 and is decreased when MeCP2 is KO (Hansen, Sakamoto, Wayman, Impey, & Obrietan, 2010; Im, Hollander, Bali, & Kenny, 2010; Klein et al., 2007; Tognini, Putignano, Coatti, & Pizzorusso, 2011).

In fact, miRNA-132 expression seems to affects synaptic transmission, which in turn controls neuronal circuits, and the tight regulation and fine-tuning of these processes is essential for brain function. In the light of that, miRNA-132 was reported to be upregulated in the mouse hippocampus after presentation of spatial learning tasks (Hansen et al., 2010) and diminished in schizophrenic patients, where, in tissue from adult schizophrenic subjects, altered expression of several miRNA-132 targets (DNMT3A, GATA2, and DPYSL3) was reported (Mellios & Sur, 2012; Miller et al., 2012).

Regarding its function in local protein translation, miRNA-132 is preferentially enriched at synapses or dendrites, compared with the perinuclear cell soma in mature neurons, although in dorsal root ganglion neurons from midgestation embryos this miRNA is mostly present in axons. Hence, subcellular localization of this miRNA is dependent on development stage or/and neuron type (Hancock et al., 2014). Albeit, the molecular mechanism of transport of miRNA is unknown, it is speculated that RNA-binding proteins, such as FMRP, deliver or anchor the miRNA to dendrites (Edbauer et al., 2010; O'Carroll & Schaefer, 2012). Interestingly, RNA-binding proteins have been found to create a feedback loop to inhibit miRNA ensuing in a homeostatic role in miRNA regulation (B. Ryan et al., 2015a).

Taken together, the existing evidence suggests that the miRNA-132 pathway is modulates dendritic morphogenesis and structural plasticity associated with long-term facilitation in the hippocampus (Wayman et al., 2008). However, further studies are needed to elucidate the whole regulatory pathways and how the activity-dependent alterations occur in other brain structures (Magill et al., 2010).

These findings highlight the importance of restrict miRNA-132 levels over a fine concentration range, since once dysregulated, its expression could ultimately impair signalling mechanisms that are involved in learning and memory formation (Hansen et al., 2016; Rajgor & Hanley, 2016).

miRNA-186

Little is known and described in the literature about miRNA-186. Most of the performed studies so far focus their attention on the function of this miRNA in cancer, various forms of cardiovascular disease, glomerulosclerosis, as a biomarker to evaluate disease activity, as well as in muscle cell differentiation. However, Tsang and colleagues have shown that miRNA-186, among others, has a positive correlation for transcripts present in neuronal cells, in other words, an increase in the miRNA level coincides with a decrease in the levels of its target transcripts at the tissue level (Lau et al., 2008; Tsang, Zhu, & van Oudenaarden, 2007). Moreover, recently miRNA-186 was described to be enriched in neurons (Jaekwang Kim et al., 2016). In 2008, a study by Zhou and colleagues provided the first evidence of miRNA-186 involvement in receptors regulation (L. Zhou, Qi, Potashkin, Abdul-Karim, & Gorodeski, 2008). Authors found a putative target sites for miRNA-186 within the 3'UTR of P2X7, a purinergic receptor. P2X7 was validated as a target of miRNA-186using a luciferase reporter containing the full-length human 3'UTR-P2X7 and treatment with miR-186 inhibitors. These data suggest that miRNA-186 targets P2X7 3'UTR and regulates its levels.

In chronic stress studies, microarray analysis identified miRNA-186 as being upregulated in cerebellum, extending the analysis later to hippocampus and prefrontal cortex. Subsequently, epidermal growth factor (EGF) receptor pathway substrate 15 (Eps15), believed to coordinate clathrin-mediated endocytosis, was shown to be a target of this miRNA (Babenko, Golubov, Ilnytskyy, Kovalchuk, & Metz, 2012).

Although miRNA-186 has not been clearly linked to Alzheimer disease (AD), its levels gradually decrease during aging, in cortex (Kim et al., 2016). Moreover, studies have identified it as regulator of nicastrin (NSCTN) and APP-cleaving enzyme 1 (BACE1) (Delay et al., 2014; Jaekwang Kim et al., 2016), proteins involved in the mechanisms of AD. BACE1 was validated as a target of miRNA-186; BACE1 and miRNA-186 levels have an inverse correlation during aging and upon overexpression of miRNA-186 there is a reduction in BACE1 levels and Aβ secretion (Jaekwang Kim et al., 2016). Likewise, recent evidence suggests miRNA-186 represses both mature and immature forms of NSCTN, which could decrease Aβ generation at both β - and γ-cleavage steps (Delay et al., 2014). Notably and interestingly, P2RX7, known to increase cleavage of APP *in vitro,* are elevated in AD brains (Delarasse, Auger, Gonnord, Fontaine, & Kanellopoulos, 2011; McLarnon, Ryu, Walker, & Choi, 2006). Together, these finding suggest that miRNA-186 may play an important role in early onset mechanisms of the AD (Jaekwang Kim et al., 2016).

miRNA-186 has also been linked to drug addiction, alcohol exposure and withdrawal. Regarding drug addiction studies, a screening of altered miRNAs in prefrontal cortex (PFC) of rats that consumed methamphetamine showed increased levels of miRNA-186 expression (Du et al., 2016).

Bekdash and Harrison have reported that both chronic exposure to alcohol and alcohol withdrawal upregulate miRNA-186 levels. Subsequently, α4 subunit of $GABA_A$ receptors (*Gabra4*) was validated as a target of miRNA-186, with its expression levels inversely correlated with miRNA-186 levels in these treatments and also in overexpression or downregulation of this miRNA levels (Bekdash & Harrison, 2015).

Preliminary supporting evidences

Preliminary data from our group demonstrated that miRNA-186 levels are downregulated with neuronal activity blockade in hippocampal neurons and that this miRNA is involved in synaptic processes, targeting the transcripts for synaptic proteins.

Primary cultures of rat hippocampal neurons were subjected to chronic neuronal activity blockade with GYKI and MK801 (antagonists of AMPARs and NMDARs, respectively) for 9h. These samples were then subjected to Agilent gene expression microarray in order to assess alterations in gene expression (unpublished data, work performed by Joana Fernandes). Gene ontology analysis of altered gene expression was performed using GoMiner™ software (version 12.9.0) and 8 large categories of altered transcripts were selected to predict putative miRNA regulators of these altered genes, using three different algorithms: miRanda (August 2010 Release, updated in November 2010), TargetScan (Version 6.1 released in March 2012) and MirTarget2 (Updated in April 2012). Eventually this analysis rendered about 600 putative miRNA regulators. Among these, we selected 7 altered transcripts with known synaptic function: CAMKIIB, CNTNAP1, GRIN1, NLNG1, which were upregulated; and Arc, BDNF and PLK2, which were downregulated in the microarray analysis, and selected 22 miRNAs for the screening panel based on their prediction score, other relevant putative targets with a synaptic function, and the fact that they were predicted by more than one bioinformatic tool (Figure 13; unpublished data, work performed by Mariline Silva).

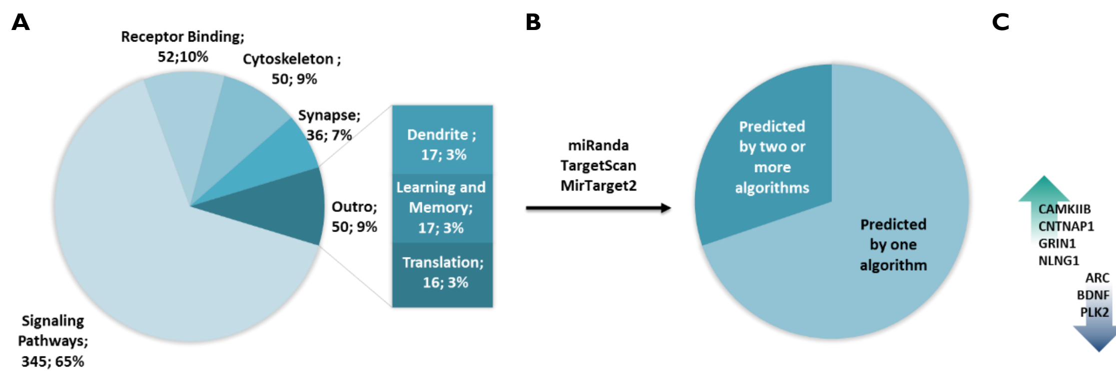

Figure 13| MicroRNA prediction based on genomic response to blockade of activity. RNA was extracted from rat hippocampal neurons (15 DIV) subjected to GYKI and MK801 treatment for 9h, and Agilent gene expression microarray analysis was performed. About 30.370 transcripts were detected in the hippocampal cultures. Student's t-tests were used to determine the genes whose expression was significantly different between the blockade of activity treatment and the correspondent control. The thresholds were 2.0 for fold change and p<0.05 for the p-value, resulting in 965 genes with altered expression. Unpublished data, work performed by Joana Fernandes. A. Gene ontology analyses were performed using GoMiner tool and several gene categories relevant for synaptic events were selected, which accounted for 45.2% of the genes that presented altered activity in the microarray analysis. B. miRNA target sites were predicted in the selected gene categories using three different algorithms: miRanda, TargetScan and MirTarget2. 181 miRNAs were selected, identified by at least two different algorithms. C. Based on a restricted group of altered genes with a crucial role in synaptic scaling events (green for upregulation and blue for downregulation), miRNA target scores for each site and other putative targets, the miRNA panel was reduced to 18 miRNAs. Unpublished data, work performed by Mariline Silva.

The screening panel evaluated the expression of miRNAs within several periods of chronic activity blockade (2h, 4h, 9h and 24 h). Interestingly, miRNA-186 is negatively regulated upon 2 h of neuronal activity blockade (unpublished data, work performed by Mariline Silva).

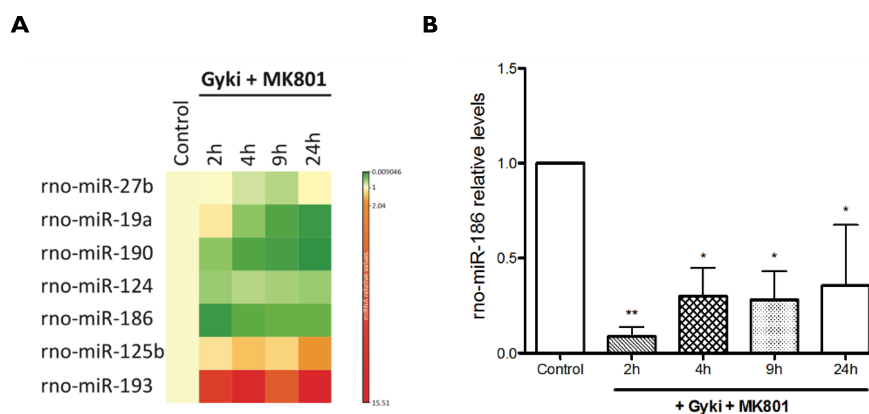

Figure 14| Activity blockade modulates miRNA levels in hippocampal neurons. Rat hippocampal neurons (15 DIV) were subjected to GYKI and MK801 treatment for 24 h, 9 h, 4 h and 2h. RNA was extracted and Pick-and-Mix PCR panels design based in the predicted miRNAs were performed. **(A)** Heat map representation of miRNA relative levels of miRNA-19a, miR-27b, miR-186, miR-190, miR-124, miR-125b and miR-193 with neuronal activity blockade for 2h, 4h, 9h and 24h. **(B)** miRNA-186 relative levels decrease significantly with activity blockade. Results represent four independent experiments and presented as means ± S.E.M. Statistical significance was determined by ANOVA followed by Dunnett's Multiple Comparison Test (*p<0.05, **p<0.01), relative to control. Unpublished data, work performed by Mariline Silva.

Since this miRNA levels were hugely downregulated after 2 h, *in silico* analysis was performed and several interesting putative targets were identified, such as GluA2 and GluA3 subunits of AMPARs,

GluN3A subunit of NMDARs, Shank2 and Caspr1. GluA2 was the first target to be validated due to its crucial in synaptic plasticity mechanisms and, since the putative binding site of this miRNAS was highly conserved in mammals (Figure 15.A). Luciferase assay indicates that GluA2 3'UTR is regulated by miRNA-186 (Figure 15.B). Furthermore, GluA2 synaptic levels are affected on opposite directions when miRNA-186 is overexpressed or inhibited (Figure 15.C), confirming the GluA2 regulation by miRNA-186 (unpublished data, work performed by Mariline Silva).

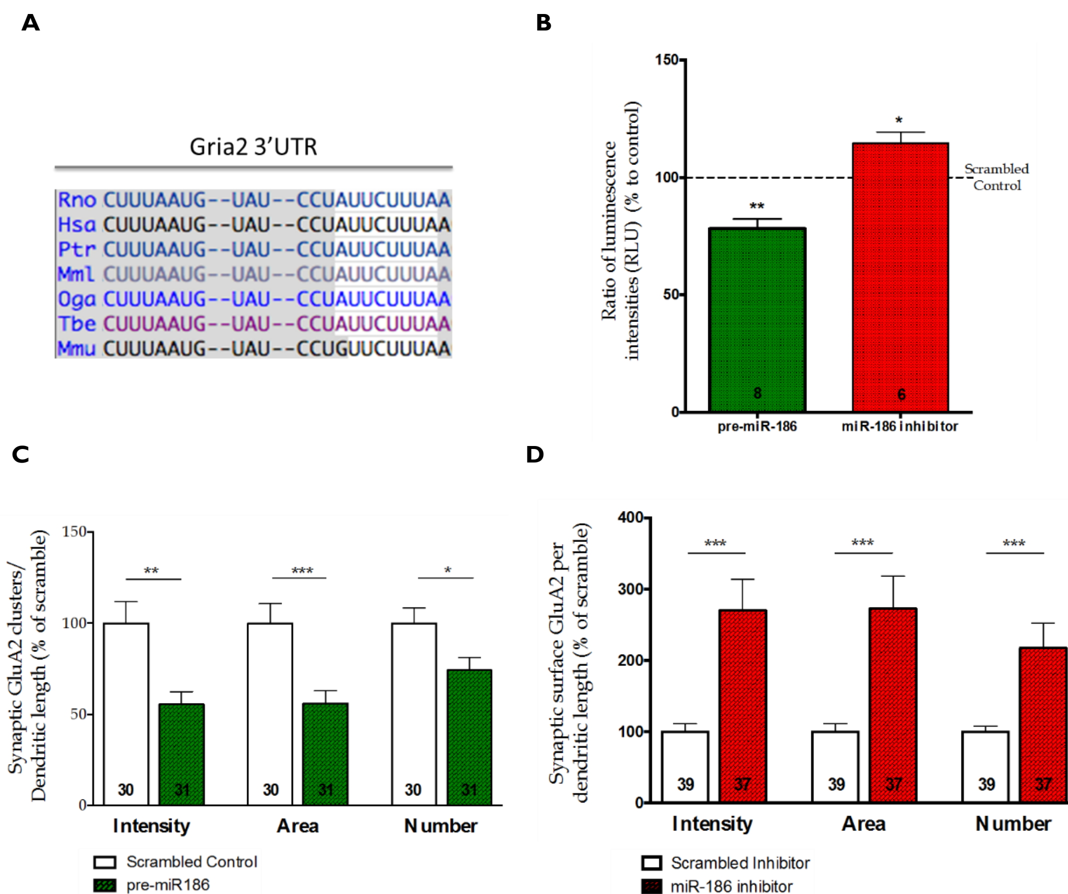

Figure 15| GluA2 is a target of miR-186. A. The putative binding site for the seed sequence for miRNA-186 (highlighted) in the 3'UTR of GluA2 is highly conserved among mammals and primates. **B.** Relative luciferase activity of a reporter gene (gaussia luciferase) fused with GluA2 3'UTR co-transfected with Scramble, pre-miR-186 or mir-186 inhibitor. Luciferase activity normalized to SEAP activity. Results from two independent experiments. Error bars, ± S.E.M. **p<0.01, t-test. **C-D.** Quantification of synaptic GluA2 clusters (co-localized with VGLUT1) shows that miRNA-186 overexpression (**C**) or inhibition (**D**) promotes an up- and downregulation of GluA2 protein levels, respectively. Results from four independent experiments. Error bars, ± S.E.M. *p<0.05 and **p<0.01, *t-test*. Unpublished data, work performed by Mariline Silva.

Therefore, all these evidences prompt us to believe that miRNA-186 can potentially be a crucial regulator of synaptic plasticity mechanisms and it is important to further characterize its role in processes like neuronal development, synaptic plasticity and synaptic transmission.

Objectives

It is widely known that the miRNA system regulates neuronal mechanisms highly dependent of protein synthesis such as neurodevelopment processes and synaptic plasticity. In fact, KO and KD approaches demonstrate the fundamental role of miRNAs on development (Petri et al., 2014b; Ziats & Rennert, 2014a). Moreover, miRNAs can be regulated by neuronal activity at several levels, from regulation of their transcription by transcription factors like CREB and MeF2 that respond to neuronal activity, to their degradation (G. Schratt, 2009). Therefore, among several targets processes and mechanisms regulated by miRNAs, dendritic growth and remodelling through the influence on cytoskeleton dynamic by target actin polymerization regulator proteins (G. M. Schratt et al., 2006; Sim et al., 2014) and regulation of synaptic strength via direct interference with AMPARs or indirectly regulating proteins that have an effect on surface amount of these receptors (Cohen et al., 2011; Gu et al., 2015; Letellier et al., 2014) have already been described.

Previous results from our group led to the identification of miRNA-186 and miRNA-132 as neuronal-activity regulated miRNAs. In fact, neuronal activity blockade in hippocampal neurons rapidly decreases the levels of both these miRNAs. Moreover, *in silico* analysis uncovered the GluA2 subunit of AMPARs as a putative target of miRNA-186 and miRNA-132, which is being currently validated in *in vitro* studies.

Therefore, our main goal is to further characterize these two miRNAs in the regulation of synaptic function, by pursuing the following objectives:

1) Assessing the levels of miRNA-186 and miRNA-132 throughout development and during maturation; since GluA2 is a predicted target for both miRNA-132 and miRNA-186, variations in the levels of both miRNAs are correlated with GluA2 levels.

2) Evaluating the impact of neuronal activity manipulation paradigms in hippocampal neuronal cultures on the expression level of miRNA-186 and miRNA-132. Hence, chemical-LTP protocol, depolarization-induced sustained activation using KCl and two different protocols for LTD induction, dependent and independent of protein synthesis are applied on hippocampal dense cultures. These plasticity protocols are validated, and their effect on the miRNAs levels are determined.

3) Testing whether miRNA-186 affects excitatory synaptic transmission in hippocampal neurons. In fact, our data indicate that miRNA-186 regulates GluA2 levels in basal conditions, hinting for a possible role of this miRNA in the regulation of synaptic strength. Whole-cell patch clamp methods are applied to evaluate the frequency and amplitude of mEPSCs in neurons whose levels of miRNA-186 are manipulated,

Overall, these analyses will clarify the importance of miRNA-186 and miRNA-132 in synaptic plasticity by possibly determining the involvement of these miRNAs on neuronal development and

synaptic strength modulation. Furthermore, the results obtained will be relevant to understand how neuronal activity regulates the expression of these two miRNAs and unveil possible molecular mechanisms that regulate the GluA2 subunit of AMPARs relying on the miRNA system.

Material & Methods

Materials

Materials

Minimum essential medium Eagle (MEM), Kynurenic acid as well as the protease inhibitors chymostatin, leupeptin, antipain and pepstatin (CLAP, stock solution 1mg/ml in dimethyl sulfoxide – DMSO) and Ponceau S stainnng solution were purchased from Sigma-Aldrich Química S.A. (Sintra, Portugal). Neurocult SM1 neuronal supplement was acquired from Stemcell Technologies (Grenoble, France). Neurobasal medium (NBM), horse serum (HS), penicillin-streptomycin (Pen-Strep), trypsin and gentamycin were purchased from Gibco, as part of Invitrogen Life Technologies (Barcelone, Spain). The BCA assay kit was purchased from Pierce, as part of Thermo Fisher Scientific (Rockford, Illinois, USA). The enhanced chemifluorescence substrate (ECF) was acquired from Amersham, as part of GE HealthCare (Carnaxide, Portugal). The fluorescent mounting medium was from DAKO (Glostrup, Denmark). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (USA). Denaturating buffer, first Strand cDNA Synthesis Kit for RT–PCR for mRNA as well as the NZYMidiprep kit were obtained from NZYTech (Portugal). miRNACURY RNA Isolation Kit – cell & plant, lysis additive, universal cDNA synthesis kit II and LNA™ PCR primer set for miRNAs, as well as ExiLENT SYBR Green master mix were purchased from Exiqon (Denmark). SsoFat™ Evagreen® Supermix was acquired from Bio-Rad (Amadora, Portugal). TTX, AP-V, and Picrotoxin were purchased from Tocris Bioscience (Bristol, UK). Glycine was purchased from Fisher Scientific (Lisboa, Portugal). Potassium chloride was acquired from José M. Vaz Pereira (Benavente, Portugal).

All other chemical reagents were obtained from Sigma (Sintra, Portugal), Fisher Scientific and NZYTech (Portugal).

Plasmids/constructs

Both the miRNA-186 inhibitor (Figure 16) and the scrambled inhibitor control constructs (pEZX-AM04 vector) were purchased from GeneCopoeia (Rockville, MD).

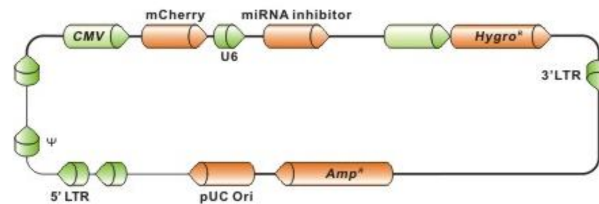

Figure 16| Map of the pEZX-AM04 (HIV based) vector.

Antibodies

For immunocytochemistry experiments the following primary and secondary antibodies were used: anti-GluA1 N-terminal (GR1 residues 271-285 of rat S453B 2nd Bleed, sheep; kindly offered by Dr.Andrew Irving – University of Dundee); anti-VGluT1 (anti-Vesicular Glutamate transporter 1, Poly clonal antibody, Guinea Pig, Millipore) and anti-MAP2 (anti-MAP2 antibody, rabbit, Sigma-Aldrich). AlexaFluor 488 antibody (donkey anti-sheep IgG, Invitrogen); AlexaFluor 647, (goat anti-Guinea Pig IgG, Invitrogen) and AMCA (AffiniPure Goat Anti-chicken IgY (IgG, Jackson ImmunoReseArch)).

For western blot experiments following primary and secondary antibodies were used: anti-GluA2, (anti-Glutamate receptor 2, extracellular, clone 6C4, mouse, Millipore) and Alkaline phosphatase-conjugated IgG secondary antibody (anti-mouse, Jackson ImmunoReseArch).

Methods

Hippocampal cultures (low and high density cultures)

The low-density cultures were prepared based on (Banker & Goslin, 1998). Hippocampi of E17-E19 Wistar rat embryos were dissected, washed twice with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red) and chemically dissociated using trypsin (0.25%) in HBSS for 15 minutes at 37°C. To stop trypsin activity, hippocampal cells were washed six times with HBSS. Finally, neurons were mechanically dissociated, andplated in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid) onto poly-D-lysine-coated coverslips or plates (0.1 mg/ml). The final cell density was 3 x 10^5 cells/60 mm culture dish for immunocytochemistry; 90000 cells/cm^2 in 6-well plates for biochemical purposes; or 80 000 cells/cm^2 in 12-well plates containing 18 mm coverslips for electrophysiological studies. After 2 h of incubation at 37°C in a humidified incubator of 5% CO_2/95% air, coverslips of low-density cultures (Banker cultures) were flipped over an astroglial feeder layer in Neurobasal medium [supplemented with SM1 neuronal supplement (1:50 dilution; StemCell technologies), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin]; in the high density cultures the plating medium was replaced for supplemented Neurobasal medium. In Banker cultures wax dots on the neuronal side of the coverslips allowed the physical separation of neurons from glia, despite neurons growing face down over the feeder layer. To further prevent glia overgrowth, high and low density neuronal cultures were treated with 5 μM cytosine arabinoside after 3 days *in vitro* (DIV) (Meyers et al., 2005), excluding neurons for biochemical studies. Cultures were maintained at 37°C in a humidified incubator of 5% CO2 /95% air, up to 28 days. The medium of all the cultures was replenished every 3-4 days, starting at 7 DIV, with fresh Neurobasal medium without glutamate.

Neuron transfection with the calcium phosphate protocol

Constructs were recombinantly expressed in both high- and low-density primary cultures of hippocampal neurons at 10-11 DIV, using a calcium phosphate transfection protocol adapted from Jiang and collaborators (Jiang, Deng, & Chen, 2004). Prior to DNA precipitate addition, neurons were pre-treated with 2 mM kynuneric acid in conditioned Neurobasal medium, during 20 minutes. DNA precipitates were prepared by diluting plasmid DNAs (2-2.5 μg *per* coverslip) in Tris-EDTA transfection buffer (10 mM Tris-HCl and 2.5 mM EDTA, pH 7.3), followed by the addition of

$CaCl_2$ solution (2.5 M in 10 mM HEPES) drop-wise to the diluted DNA, to a final concentration of 250 mM $CaCl_2$. The DNA-$CaCl_2$ mix was then added drop-wise to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, and 42 mM HEPES, pH 7.2), thoroughly mixed, leaving the precipitate to develop at room temperature for 5-10 minutes.

The DNA precipitates were added, drop-wise, to each coverslip, and the neurons were incubated with the precipitates approximately for 2 h (in the presence of kynurenic acid), in order to allow the DNA precipitates internalization via endocytosis. Then, the remaining precipitates were dissolved by replacing the medium with new pre-warmed supplemented neurobasal medium (without glutamate) with 2mM kynurenic acid, slightly acidified with HCl (~5 mM final concentration) for 15 to 20 min at 37°C. Finally, each coverslip was transferred to the original dish/plate containing the conditioned medium and returned to a 37°C and 5% CO_2/95% air incubator to allow expression of the transfected constructs, until 15-16 DIV.

Chemical protocol of Long-term potentiation

For cLTP experiments, 3 different protocols were performed in high-density neuronal cultures to select the most effective approach to induce LTP. For the first protocol, neurons at 21 DIV were pre-incubated for 15 min at 37 °C, 5% CO_2/95% air incubator in chemical LTP buffer (125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 33 mM glucose, 25 mM HEPES [pH 7.4], 0.1 μM picrotoxin, 500 nM TTx, 1 μM strychnine), followed by glycine treatment for 4 min at room temperature (cLTP buffer, 200 μM glycine, without magnesium), and then returned to the original buffer (without glycine) to recover for 20 or 60 min prior to RNA extraction. For the second protocol, 21 DIV high density cultures were subject to chemical protocol of LTP with glycine in a magnesium-free medium, adapted from Ribeiro et al., 2014. Briefly, cells were washed two times with extracellular solution (ECS) containing: 150 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES, 30 mM Glucose, 0.001 mM TTX, 0.01 mM strychnine, 0.1 mM picrotoxin, pH 7.4. After washing, neurons were stimulated with 200 μM glycine at room temperature for 3 min in ECS and then washed one more time. Subsequently, neurons recovered for 0, 20 and 60 min in ECS (without glycine) in a 37°C, 5% CO_2/95% air incubator. For the third approach, the second protocol was modified, only by increasing the glycine concentration to 300 μM (freshly prepared), to ensure LTP induction on hippocampal cultures. For each recovery time point, there is a control well that was not stimulated with glycine, in order to normalize the respective condition and minimize possible variations on the stimulations and recovery times between control and stimulated conditions. Afterward, total RNA was isolated from the neuronal cultures.

Chemical protocol of Long-term depression dependent of NMDA receptors

To achieve NMDAR-mediated LTD, 16 DIV high density cultures were treated with 20 µM NMDA for 3 min at room temperature, as described by (H. Lee, Lee, Song, & Kim, 2014). Control condition had no NMDA supplementation. After stimulation, cultures were washed with new pre-warmed supplemented neurobasal medium without glutamate, and posteriorly incubated in the remaining conditioned medium for 20 and 60 min, to allow recovery, in a 37 °C, 5% CO_2/95% air incubator. Then, RNA was isolated from the neuronal cultures.

The same approach was also performed on 16 DIV Banker cultures transfected with scrambled control plasmid for miRNA inhibition, in order to assess if this protocol was effective in transfected neurons. Coverslips were transferred to a 12-well plate and incubated for 3 min in conditioned medium containing 20 µM NMDA at room temperature. Neurons were then washed with new pre-warmed supplemented neurobasal medium without glutamate and relocated to the original dish with conditioned medium, returning to a 37°C and 5% CO_2/95% air incubator for 20 and 60 min of recovery. Subsequently, immunocytochemistry was performed.

Chemical protocol of Long-term depression dependent of group I mGluRs

To trigger mGluR-dependent LTD, 16 DIV high density cultures were incubated with 100 µM DHPG, a group 1 mGluR agonist, for 5 min (Jakkamsetti et al., 2013; S. Park et al., 2008; Snyder et al., 2001; Waung, Pfeiffer, Nosyreva, Ronesi, & Kimberly, 2009). Control condition was incubated on a solution with no drug addiction. After stimulation, neuronal cultures were washed with new pre-warmed supplemented neurobasal medium without glutamate, and recovered in the remaining conditioned medium for 20 and 60 min at 37°C and 5% CO_2/95% air. Finally, RNA was isolated from neuronal cultures.

Stimulation to increase neuronal activity of hippocampal cultures

Activity in 16 DIV high density hippocampal neurons was enhanced with the chronic application of 16 mM KCl in conditioned medium for 6 h or 25 mM for 1 h and 2 h, with no recovery time, at 37°C and 5% CO_2/95% air. The control well was not stimulated. After the chronic incubation period, RNA was extracted from these cultures.

RNA extraction of primary hippocampal cultures

Total RNA was isolated from high-density primary hippocampal cultures (6-well plates) using miRNACURY RNA Isolation Kit – cell & plant (Exiqon), following the protocol provided by the manufacturer.

Briefly, neurons were lysed with the Lysis solution, supplemented with β-mercaptoethanol (Life Technologies), for 5 min. Lysates were scraped and transferred to a microcentrifuge tube and 96-100% ethanol was added, followed by a short vortex. Next, RNA containing solution was transferred onto a column with high affinity for RNA, where the RNA was purified and washed with Wash Solution. Afterwards, the column resin was dried by centrifugation and RNA was eluted with the Elution Buffer. To increase RNA recovery, the elution step was repeated, applying the RNA eluted again onto the column. RNA concentration was measured by spectrophotometry using NanoDrop Lite spectrophotometer (Thermo Scientific).

RNA extraction of hippocampus tissue

Hippocampi of P1, P8, P15, P22, P29, P36 and P43 animals were snap frozen with liquid nitrogen and stored at -80°C upon dissection and later the RNA was isolated using miRNACURY RNA Isolation Kit – cell & plant (Exiqon) following the adapted protocol for tissues provided by the manufacturer. Briefly, tissue was transfer to a mortar in dry ice and reduced to powder by grinding it with the help of a pestle, without allowing the sample to thaw. Then, the Lysis Solution (supplemented with β-mercaptoethanol) was added to the tissue and the sample homogenate was passed 5-10 times through a 25 gauge needle attached to a syringe, in order to lysate the neurons and shear the genomic DNA. Then, Lysis Additive was added to the lysate and mixed by vortexing. To further remove genomic DNA, the entire homogenate was applied onto a column and centrifuged, sorting the flowthrough containing the RNA. Next, Lysis Solution and 96-100% Ethanol were sequentially added to the homogenate, and mixed by vortexing. The following steps of the RNA purification using a high affinity column are in common from this point forward with the RNA extraction protocol from cultures described above.

First-strand cDNA synthesis for mRNA analysis

Synthesis of cDNA from RNA samples was performed following the protocol of NZY Frist-Strand cDNA Synthesis Kit (NZYTtech). Shortly a mix reaction containing: NZRT 2x Master Mix, NZRT Enzyme Mix, 500 ng of RNA, and DEPC-treated water was prepared on ice. After mixing gently, the samples were incubated at 25°C for 10 min, followed by an incubation at 50°C for 30 min and

finalized incubating the samples at 85°C for 5 min, in order to inactivate the reaction, and then chill at 4°C. Afterwards, NZY RNase H (*E. coli*) was added to the samples and these were incubated at 37°C during 20 min. All the incubations were performed in T100 thermal cycler (Bio-Rad) and cDNA products were stored at -20°C until further use.

Quantitative Real-time PCR for mRNA

RT q-PCR was performed with the Bio-Rad iCycler Thermal Cycler with iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad iQ 5 RTPCR QPCR), using SsoFat™ Evagreen® Supermix (Bio-Rad) for detection of cDNA. 2 µl of 80-fold dilution cDNA were used for real-time PCR reactions, except for time course hippocampal cultures samples where dilution was 1:40. A working-solution comprising forward primer, reverse primer, nuclease-free water and SsoFat™ Evagreen® Supermix (Bio-Rad) was added to template cDNA onto PCR 96-well plate. After sealing the plate with optical sealing, PCR plate was spinned down. Then the cDNA was amplified and detected with the following protocol: Enzyme activation at 95°C for 30 sec (1 cycle); denaturation at 95°C for 5 sec and annealing/extension at 50°C for 10 sec (40 cycles) and finally, melting curve step at 60-95°C, 10 sec/step (1 cycle). Samples were assayed in duplicates and at the end, using the relative standard curve method to determine cycle quantification, Arc and GluA2 values were normalized to GAPDH for each sample and the expression fold change was determined in the GenEx software. Primer sequences are found in Table 1.

Table 1| Sequences of forward and reverse primers used on quantitative Real-time PCR.

Primer	Forward primer sequence	Reverse primer sequence
GAPDH (STAB VIDA)	5’ TCC CAT TCT T…ACC TTT 3’	5’ TAG CCA TAT T… CAT ACC 3’
Arc (STAB VIDA)	5’ GAG TTC AAG AAG GAG TTT 3’	5’ CAC ATA CTG AAT GAT CTC 3’
GluA2 (STAB VIDA)	5’ GAA GCCTTG TGA CAC CAT GA 3’	5’ AGC CTT GCC TTG CTC CTC AT 3’

First-strand cDNA synthesis for miRNA analysis

cDNA was synthesized from 1 µg total RNA by reverse transcription using a Transcriptor First Strand cDNA Synthesis Kit (Exiqon).

First-strand cDNA was synthesized from 5 ng/µL total RNA mixed with a working solution containing RNA spike ins, Enzyme mix, Nuclease-free water and 5x Reaction buffer. The reaction consists of an incubation for 60 min at 42°C and an inactivation of the reverse transcriptase step where the samples were heated for 5 min at 95°C and immediately cooled to 4°C. These incubations were performed in T100 thermal cycler (Bio-Rad). At the end, the undiluted cDNA was stored in low-nucleic acid binding tubes, at -20°C, for up to 5 weeks.

Quantitative Real-time PCR for miRNA

To determine changes in mature microRNA levels by quantitative real-time PCR (RT q-PCR), cDNA was diluted 80-fold in nuclease-free water and 4 µl was used as template in RT q-PCR together with PCR master mix and PCR primer mix, as it is described on ExiLENT SYBR Green master mix kit protocol (Exiqon) provided by the manufacturer. After the PCR primer-master mix working-solution was prepared, it was added to cDNA template onto PCR 96-well plate. In order to ensure that all the reagents were mixed, a gently pipetting was performed, followed by the sealing of the plate with optical sealing. Subsequently, PCR plate was spin down. The amplification reaction was the following: a first cycle at 95°C during 10 min to activate polymerase and denaturation, followed by 45 amplification cycles at 95°C for 10 sec and posteriorly 60°C for 1 min, and finally there was optical read. This ramp-rate of cooling from 95°C to 60°C was set to 1.6°C/s. This reaction was performed on Bio-Rad iCycler Thermal Cycler with iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad iQ5 RTPCR QPCR).

Samples were assayed in duplicates and using the relative standard curve method to determine cycle quantification, miRNA-132 and miRNA-186 values were normalized to miRNA-99b for each sample and the expression fold change was determined using the GenEx software. Primer sequences are found on Table 2**.**

Table 2| Primers target sequences used on quantitative real-time PCR.

Primer	Target sequence
miRNA-99b-5p (Exiqon)	5' CAC CCG UAG AAC CGA CCU UGC G 3'
miRNA-186-5p (Exiqon)	5' CAA AGA AUU CUC CUU UUG GGC U 3'
miRNA-132-3p (Exiqon)	5' UAA CAG UCU ACA GCC AUG GUC G 3'

Immunocytochemistry – GluA1 live staining

Hippocampal neurons from Banker cultures at 16 DIV stimulated with NMDA were labelled with anti-GluA1 N-terminal 1:200 (GR1 residues 271-285 of rat S453B 2nd Bleed, sheep; kindly offered by Dr. Andrew Irving – University of Dundee) in conditioned neurobasal medium, at room temperature for 10 min. Then, neurons were fixed in 4% sucrose / 4% paraformaldehyde in PBS for 15 min and washed 6 times in PBS before being permeabilized with 0.25% Triton X-100 in PBS for 5 min, at 4°C. Subsequently, neurons were washed again with PBS and incubated in 10% (w/v) BSA in PBS for 30 min, at 37°C to block nonspecific staining. After that, coverslips were incubated with secondary antibody AlexaFluor 488 1:1000 (donkey anti-sheep IgG, Invitrogen) diluted in 3% BSA in PBS, overnight at 4°C. Then, neurons were washed thoroughly with PBS and posteriorly labelled for VGluT1 1:5000 (anti-Vesicular Glutamate transporter 1 Poly clonal antibody, Guinea Pig, Millipore) and MAP2 1:5000 (anti-MAP2 antibody, rabbit, Sigma-Aldrich) for 2 h at 37°C. Posteriorly, neurons were washed with PBS and stained with the appropriate secondary antibodies (AlexaFluor 647 Goat anti-Guinea Pig IgG, 1:1000, Invitrogen and AMCA AffiniPure Goat Anti-chicken IgY (IgG), 1:200, Jackson ImmunoReseArch), diluted in 3% BSA in PBS, for 45 min – 1 h at 37°C. Coverslips were then washed twice with 0.1% Triton X-100 in PBS, followed by four washes with PBS and finally mounted using fluorescent mounting medium from DAKO. Preparations were preserved overnight at 4°C, protected from light, sealed with nailpolish and kept at 4°C until microscopy analysis.

Fluorescence microscopy and quantification

Fluorescent imaging was performed on a Zeiss Axiovert 200M inverted microscope, with an AxioCam HRm camera and with AxioVision 4.8 software. Images were acquired with a Plan-Neofluor 63 x oil objective (numerical aperture 1.4).

Neurons were cultured, transfected and stained simultaneously and imaged using identical settings. For quantification, fields for imaging were chosen in the mCherry channel, for the presence of transfected mCherry-positive neurons. GluA1 was analysed and quantified with Image J 1.43 analysis software. Dendrites of at least 9 cells per condition were randomly chosen from exported 16-bit images and their lengths determined. Selected dendrites had similar thickness and appearance and their selection was carried out in MAP2 images, in a blind manner. GluA1 signals were analysed after thresholds were set, such that recognizable clusters were included in the analysis, and the background intensity of each image was subtracted. Then, synaptic GluA1 puncta were selected by colocalization with VGluT1. Regions around thresholded puncta were overlaid as a mask in the VGluT1 channel, and co-localization was determined. For each selected cell, intensity, area and number of total and synaptic puncta *per* dendritic length were determined. The values obtained *per* cell were normalized against the control mean of that single experiment.

Protein extracts

Rat hippocampi (P1 to P42) were homogenised with 10 volumes (10x weight) of lysis buffer RIPA (150 mM NaCl, 50 mM Tris-HCl [ph 7.4], 5 mM EDTA, 1% Triton, 0,5% DOC, 0,1% SDS, pH 7.5), supplemented with 1 mM DTT, 200 µM phenylmethylsulfonyl (PMSF), 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1µg/ml antipain, 1µg/ml pepstatin (CLAP), 1 mM sodium orthovanadate and 50 mM sodium fluoride. The lysates obtained were sonicated on ice for 50s (5 pulses of 5 seconds each) and centrifugated at 16,100 × g for 10 min at 4°C, to discard debris. The supernatant was collected and quantified using the bicinchoninic acid (BCA) assay kit (Pierce, Termo Fisher Scientific, Rockford, IL, USA). For all samples, 150 µg of protein was denatured with 5x concentrated denaturating buffer (NZYTech) and boiled for 5 min at 95°C. The protein was frozen at -20ºC, until needed.

Gel electrophoresis and Western Blotting

Protein samples were resolved on 10% polyacrylamide gels 1.5 mm thick, by SDS-PAGE in Trisglycine-SDS (TGS) buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 80-110V. For Western blot analysis, proteins were transferred onto a PVDF membrane (Millipore, USA) by electroblotting for 100 min at 300 mA and 4°C, on CAPS-MeOH. Membranes were washed once with 0.1% Tween 20 in Tris-buffered saline (TBS) (20 mM Tris, 137 mM NaCl, pH 7.6 (TBS-T)) and then blocked for 1 h at room temperature with 5% (w/v) non-fat dry milk in TBS-T, with agitation. Membranes were probed overnight at 4°C, with the primary antibody GluA2 1:1000 (anti-Glutamate receptor 2, extracellular, clone 6C4, mouse, Millipore), respectively, diluted in TBS-T

containing 5% (w/v) low-fat milk.

Following several washes with TBS-T, membranes were incubated for 45 min with alkaline phosphatase-conjugated IgG secondary antibody 1:10000 (anti-mouse, Jackson ImmunoReseArch) at room temperature, washed again and immunostained with ECF (GE Heathcare, Carnaxide, Portugal) for up to 5 min at room temperature. Membranes were scanned with the ChemiDoc Imaging System (BioRad, Amadora, Portugal). Total protein amount was labeled using Ponceau S stainnng solution for 25 min and posteriorly scanned. Bands corresponding to the GluA2 protein and total protein were quantified using Image J software and normalized to total protein amount indicated in figure captions.

mEPSC recordings and analysis

Whole-cell voltage clamp recordings were made at room temperature from 15 DIV transfected neurons plated on coverslips. The recording chamber was mounted on a fixed-stage upright microscope (Zein examiner .D1) and perfused at 0,31 mL/ min in extracellular solution (140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 4 mM $CaCl_2$, 4 mM $MgCl_2$, pH was adjusted to 7.3 and osmolarity 300-310 mOsm, supplemented with 100 µM picrotoxin (Tocris Bioscience), 500 nM TTX (Tocris Bioscience), 50 µM AP-V (Tocris Bioscience)). Fluorescent and trasmission illumination (HBO 100) and a camera (Q-imaging) were used to visualize neurons on a computer monitor using Q-capture Pro 7 software interface. Patch electrodes (3–5 mΩ) were made from borosilicate glass (Science Products, Germany) and filled with a solution composed of 107 mM $CsMeSO_3$, 10 mM CsCl, 3.7 mM NaCl, 5 mM TEA-Cl, 20 mM HEPES, 0.2 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, corrected to pH 7.3 with CsOH and osmolarity 298-300 mOsm. Cells were voltage-clamped at –70 mV and capacitances automatically compensated. Consequently, mEPSCs were amplified using Multiclamp 700B amplifier (Axon CNS), digitised through a Digidata 1550 A plus HumSilencer and acquired using Camplex 10.5 and multiclamp 700B commander software (Molecular Device). During 3 min, mEPSC events were recorded in a gap-free acquisition mode with a sampling rate of 25 kHz after signal filtering at 2.8 kHz. Access resistance (12-22 MΩ) was monitored before and after voltage clamp recordings and was not compensated for. Clampfit software was used to detect the events and quantify amplitude, frequency and decay tau of mEPSCs recordings. Only events larger that 2x the recording noise and decay tau between 2 and 30 ms were considered. For each cell the median value was obtained and averaged across all recorded cells.

Statistical analysis

Graphs and statistical analysis were performed using GraphPad Prism 6 software. Results are plotted as normalized means ± SEM and *p* value inferior to 0.05 was chosen for statistical significance. Two sample comparisons were evaluated using non-paired two-tailed *t test*. Three or more samples were evaluated using one-way ANOVA followed by Kruskal-Wallis test for multiple comparisons.

Results

miRNA-132 and miRNA-186 expression increases in the hippocampus during development

The GluA2 subunit of AMPA receptors is a predicted target of miRNA-132 and a validated target of miRNA-186, as demonstrated by work performed in our group based on luciferase assays (unpublished data, Figure 15). Therefore, we have analysed the expression pattern of these miRNAs as well as of GluA2, *in vitro* in cultured hippocampal neurons, as well as *in vivo* in the rat hippocampus. *In vitro,* the analysis was performed in hippocampal dense cultures at 7 DIV, 14 DIV, 21 DIV and 28 DIV (Figure 17) and *in vivo*, a timeline of hippocampi from rats with different postnatal days (from P1 to P43) was constructed (Figure 18). With these approaches it was possible to compare the patterns of expression between miRNAs-132 and -186 and GluA2 mRNA by qPCR, and moreover, compare expression levels of miRNA-132 and miRNA-186. *In vivo,* the RNA analysis was complemented with protein analysis by evaluating the GluA2 expression pattern during neurodevelopment, using Western blot (Figure 18).

Hence, both *in vitro* and *in vivo* at postnatal stages, the two studied miRNAs showed to be upregulated throughout development, peaking at 28 DIV and P36, respectively (Figure 17.B-E, Figure 18.B-E), although both miRNAs presented different patterns of expression in neuron cultures and *in vivo*. In primary hippocampal cultures, while miRNA-132 maintained low levels in early developmental stages and increased dramatically at 21 DIV, attaining a > 10 fold increase at 28 DIV (Figure 17.B-C), miRNA-186 showed a persistent increase throughout neuronal development, and attained a ~3 fold increase at 28 DIV when compared to its expression level at 7 DIV (Figure 17.D-E).

However, *in vivo* and for postnatal stages of development, the expression of miRNA-132 steadily increased until P36 (11.30±2.30 fold-change relatively to P1), after which miRNA-132 levels decreased (8.71±0.89 fold-change relatively to P1; 0.77 fold-change relatively to P36) (Figure 18.C-D). Regarding miRNA-186, its levels appears to be stable early in development from P1 to P22, having a tendency to increase at P29 and increasing dramatically at P36 (2.32±0,17 fold-change relatively to P1), but declining at P43 (1.76±0.21 fold-change relatively to P1; 0.76 fold-change relatively to P36) (Figure 18.E-F). Indeed, miRNA-132 expression increased ~11 fold in the peak of its expression both *in vitro* and *in vivo* relatively to the initial time point considered, a higher increase than that of miRNA-186 expression (~3 fold-change in the peak of its expression relatively to the initial time points considered).

Additionally, it is interesting to note that our results shown a decrease on GluA2 mRNA in dissociated cultures (Figure 17.A), which is not in agreement what it has been described in the literature (Orlandi et al. 2011; Pandey et al. 2015; Petralia et al. 1999; Pickard et al. 2000); however, protein levels were increased throughout development in the hippocampus of postnatal rats (Figure 18.A).

Taking into account all the data, it is possible to describe a marked increase in the expression of these miRNAs in later stages of neurodevelopment, which is accompanied by an increase in GluA2 protein levels during the same developmental period. Therefore, it is unlikely that these miRNAs are key regulators key regulators of GluA2 expression along development. It is possible that during development GluA2 mRNA levels are primarily controlled by the transcription rate of the GluA2 gene, rather than by post-transcriptional events such as those regulated by miRNAs.

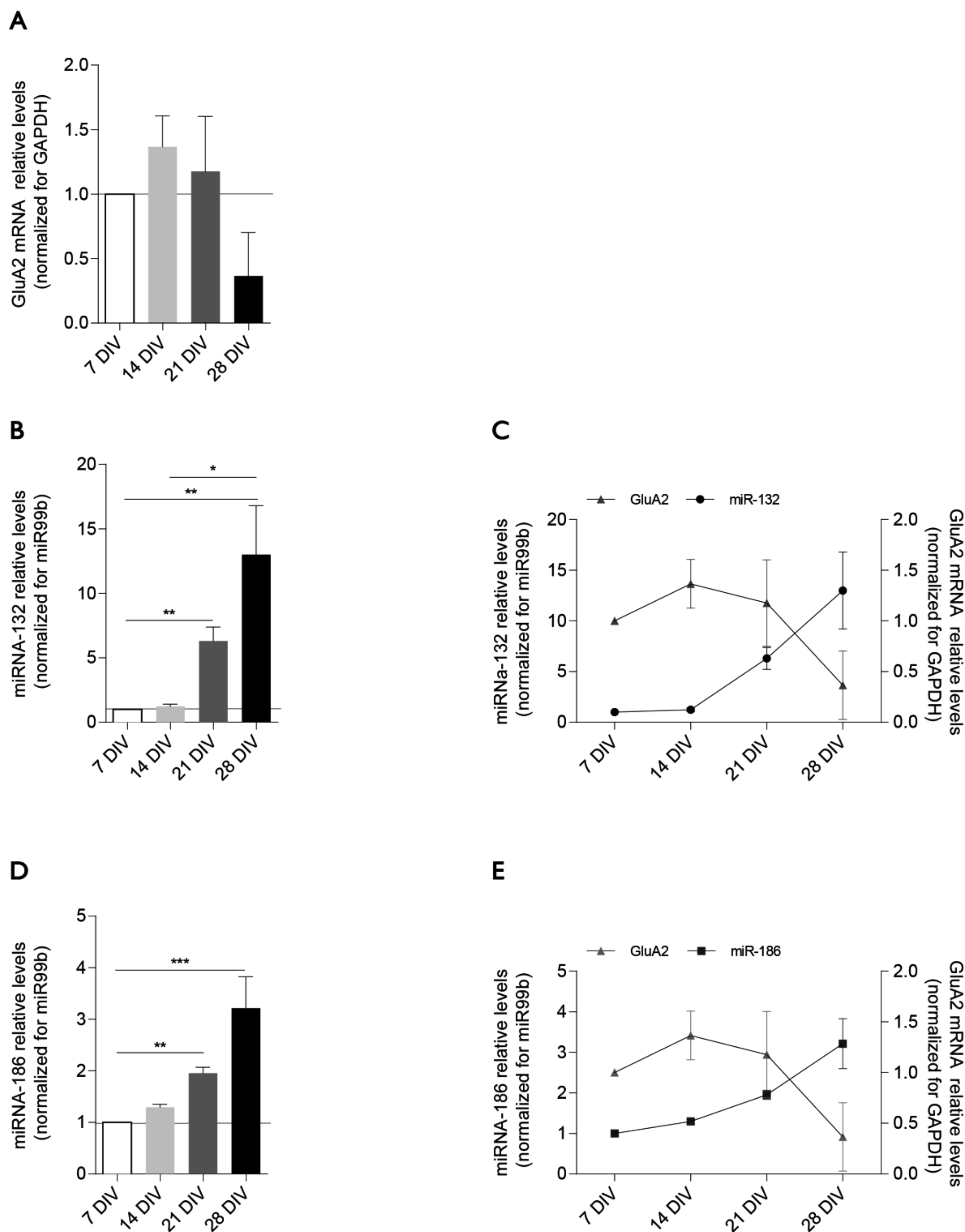

Figure 17| GluA2 mRNA expression negatively correlates with miRNA-132 and miRNA-186 expression levels in cultured hippocampal neurons during neuronal maturation. A. GluA2 mRNA levels were augmented early in development, declining slightly in the latest phases of neuron maturation that were analysed (28 DIV) (N=3). **B.** Expression of miRNA-132 is stable from 7 DIV to 14 DIV, increasing significantly at 21 DIV and 28 DIV. **C.** Inverse correlation between GluA2 mRNA and miRNA-132 expression along development; in late stages of development the decrease in GluA2 mRNA levels was accompanied by a rise in miRNA-132. **D.** Along neuronal maturation, miRNA-186 gradually increases, in particular at 21 and 28 DIV. **E.** Inverse correlation between GluA2 mRNA and miRNA-186 expression along development; in late stages of development the decrease in GluA2 mRNA levels was accompanied by a rise in miRNA-186 expression. Results are presented as means ± S.E.M., for N=6, relatively to control (7 DIV), comparing the mean rank of each group with the mean rank of every other group. GluA2 mRNA levels were normalized to the house-keeping gene GAPDH, whereas miRNA-132 and miRNA-186 levels were normalized to miRNA-99b. Statistical analysis was determined using the Kruskal-Wallis test, *$p<0.05$; **$p<0.01$ relatively to initial time point analysed (7 DIV).

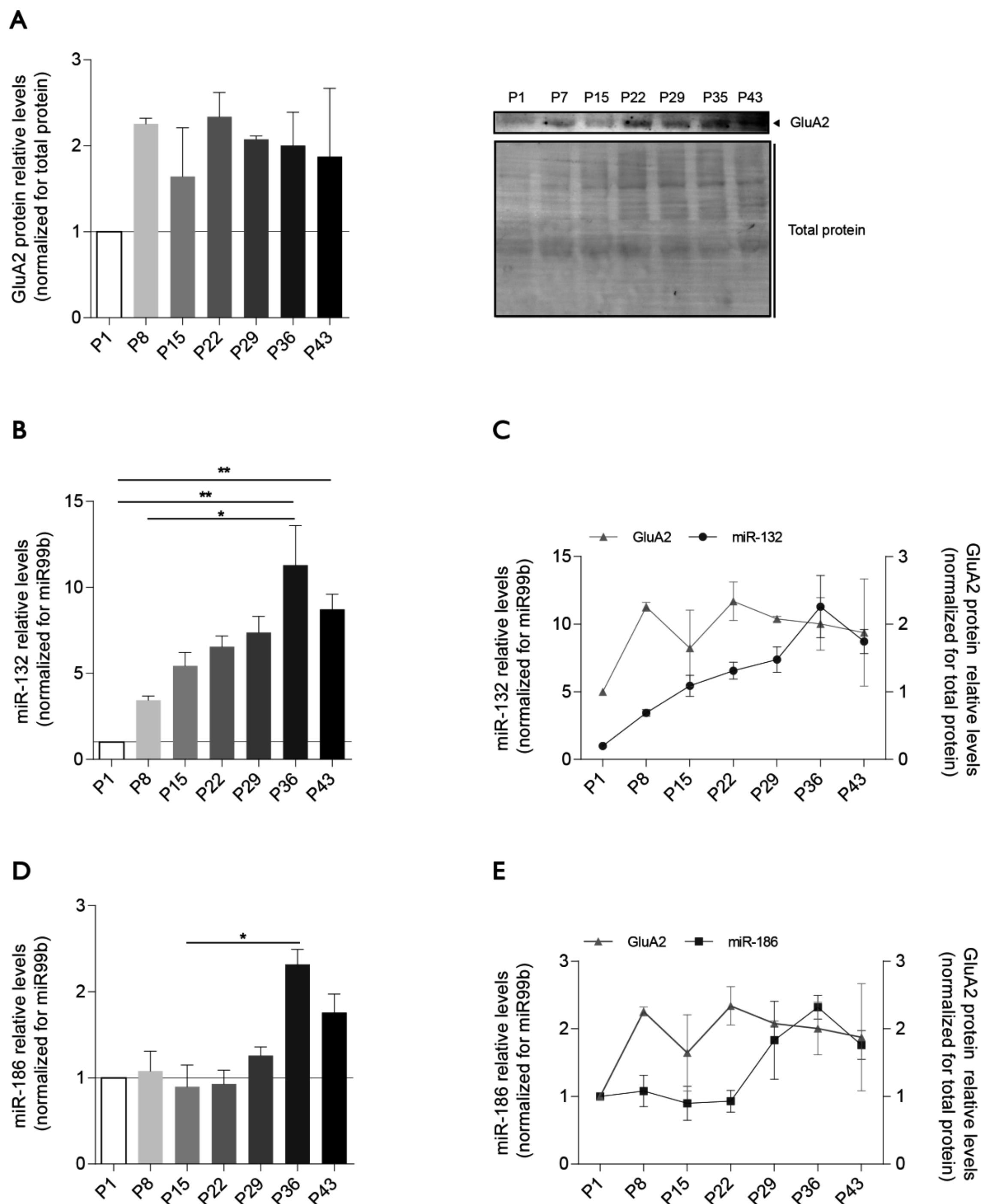

Figure 18| GluA2 protein levels and miRNA-132 and miRNA-186 expression levels throughout postnatal rat hippocampus development. A. During hippocampal development, GluA2 protein has a tendency to be increased (N=2) **B.** miRNA-132 levels increase evenly since P1, peaking at P36, and start to decrease afterwards (P43) (N=5). **C.** GluA2 protein levels and miRNA-132 expression along development. **D**. From P1 to P22, miRNA-186 levels are stable, but rise steeply thereafter, peaking at P36, and declining at P43 (N=5). **E**. GluA2 protein levels and miRNA-186 expression in the hippocampus along development. Results are presented as means ± S.E.M., relatively to the earliest time point considered (P1), comparing the mean rank of each group with the mean rank of every other group and normalized to the house-keeping miRNA-99b in the case of miRNAs and to total protein in the case of GluA2. Statistical analysis was determined using the Kruskal-Wallis test, *$p<0.05$; **$p<0.01$ relatively to the initial time point analysed (P1).

miRNA-132 levels increase upon neuronal depolarization in hippocampal neurons

Under neuronal activity blockade by Gyki and MK801, miRNA-132 and miRNA-186 are both downregulated (unpublished data from our lab), indicating that these miRNAs may be regulated during synaptic plasticity processes. To assess if neuronal depolarization also modulates these miRNA levels, high density hippocampal neurons at 16 DIV were incubated with KCl, either with 25mM KCl for 1 hour or 2 hours (Ben Fredj et al. 2004; T.-K. Kim et al. 2010) (Figure 19.A), or with 16 mM KCl for 6 hours (Fiore et al. 2009) (Figure 19.B). Afterwards, RNA was extracted and transcribed to cDNA, followed by measurement of miRNA-186 and miRNA-132 levels, by q-PCR, and comparison of their expression between stimulated and non-stimulated cells (Figure 19.D-E).

Since Arc was been reported to be upregulated under enhanced neuronal activity (T.-K. Kim et al. 2010; O'Mahony et al. 2006), both protocols of enhanced depolarization were validate by analysis of Arc expression through q-PCR technique (Figure 19.C). Arc mRNA levels were, in fact, markedly increased with 1 h or 2 h of stimulation with 25 mM KCl, but its levels were not altered after 6 h stimulation with 16 mM KCl concentration (Figure 19.C).

MiRNA-132 and miRNA-186 level measurements revealed alterations on their expression upon depolarization with KCl. Notably, miRNA-132 showed a tendency to transiently increase its expression levels with 2 h of enhanced depolarization (Figure 19.D). On the contrary, miRNA-186 expression is high variable in in our samples for the earlier time points, so no conclusions can be drawn for this part until more experiments are performed, but upon 6 h of stimulation no significant effect was observed (Figure 19.E).

Hence, sustained activation of neuronal activity may induce upregulation of miRNA-132 expression levels.

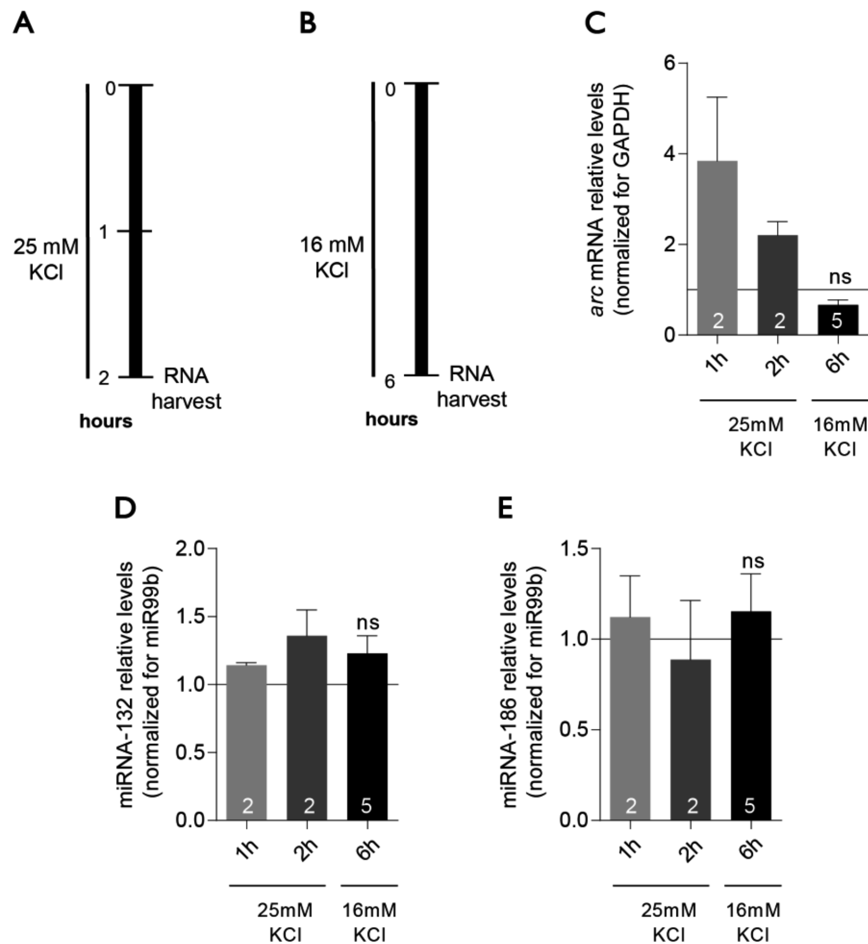

Figure 19| miRNA-132 expression levels tend to be upregulated transiently with neuronal depolarization. Arc mRNA and miRNA relative levels after 1 h, 2 h and 6 h of incubation with KCl (25 mM or 16 mM, as indicated) in 16 DIV dense hippocampal cultures, in comparison to control neurons. **A-B.** Schematic representation of the KCl-induced depolarization stimuli applied. **C.** Arc mRNA levels markedly increased upon 1 h of KCl treatment, declining afterwards (N=2), to not statistically significant levels after 6 h (N=5). **D.** miRNA-132 expression had a tendency to increase until 2 h of (25 mM) KCl-induced depolarization (N=2), although this result is not statistically significant after 6 h of treatment (16 mM) (N=5). **E.** miRNA-186 levels were not significantly altered in comparison with control condition by KCl stimulation (N=5). Results are expressed as fold change relatively to control conditions, and are presented as means ± S.E.M., when compared to control and normalized to house-keeping gene GAPDH (**C**) and miRNA-99b (**D-E**). Statistical analysis was determined by Mann-Whitney test.

miRNA-132 levels tend to be upregulated following cLTP, with no changes on miRNA-186 levels

Long-term potentiation is one of the most studied paradigms associated with learning and memory, in which several miRNAs have already implicated (Abbas, Villers, and Ris 2015; Bassani et al. 2013; Malinow and Malenka 2002). Therefore, in order to address if miRNA-132 and miRNA-186 expression levels vary in response to induction of long-term potentiation, 21 DIV high density hippocampal cultures were treated with protocols based on stimulation with glycine, an agonist of NMDA receptors, in the absence of magnesium that allows the immediate influx of calcium through NMDA receptors, as previously described (Bassani et al. 2013; Lisman, Yasuda, and Raghavachari 2012; Lu and Malenka 2012). In order to use a protocol that promoted robust and consistent LTP, we compared 3 different protocols (one of them based on neuron pre-incubation in chemical LTP buffer without magnesium followed by glycine treatment for 4 mim; in the other two, different concentrations and time of glycine stimulation in a magnesium-free medium were performed, without pre-incubation in a Mg^{2+} free-medium), followed by analysis of Arc mRNA levels, described to be increased in early LTP (Bramham et al. 2010; Orlandi et al. 2011; Shepherd and Bear 2011). To do so, after 0, 20 and 60 min of recovery upon LTP induction, RNA was extracted and mRNA levels were determined by qPCR (Figure 20).

Arc mRNA analysis in cultures submitted to the three tested LTP protocols showed that only the protocol using 3 min stimulation with 300 µM glycine induced Arc upregulation, consistent with what has been described in the literature (Figure 20**.**A). In fact, although the effects are not visible immediately after the glycine stimulus (3 min, 300 µM), after 20 min of recovery mRNA levels of Arc present 1.54±0.51 fold-increase relatively to control condition, returning to basal amounts 1h post stimulus (Figure 20**.**A). Thus, this protocol was selected to perform miRNA analysis.

Upon LTP induction, miRNA-132 expression presents a tendency to be increased immediately after the 3 min period incubation in glycine (1.36±0.16 fold-change), remaining augmented for the rest of the time to recovery (1.18±0.16 fold-change relatively to control for 20 min and 1.27±0.12 fold-change relatively to control for 60 min), albeit changes were not statistically significant (Figure 20**.**C). Moreover, LTP induction had no effects on miRNA-186 expression (0.94±0.07 fold-change relatively to control for 0 min, 0.93±0.15 fold-change relatively to control for 20 min and 1.09±0.13 fold-change relatively to control for 60 min) (Figure 20**.**D).

Therefore, LTP promotes an upregulation of miRNA-132 expression, consistent with the literature (Eacker et al. 2011; Pai et al. 2014; Wibrand et al. 2010), but it has no effect on miRNA-186 levels.

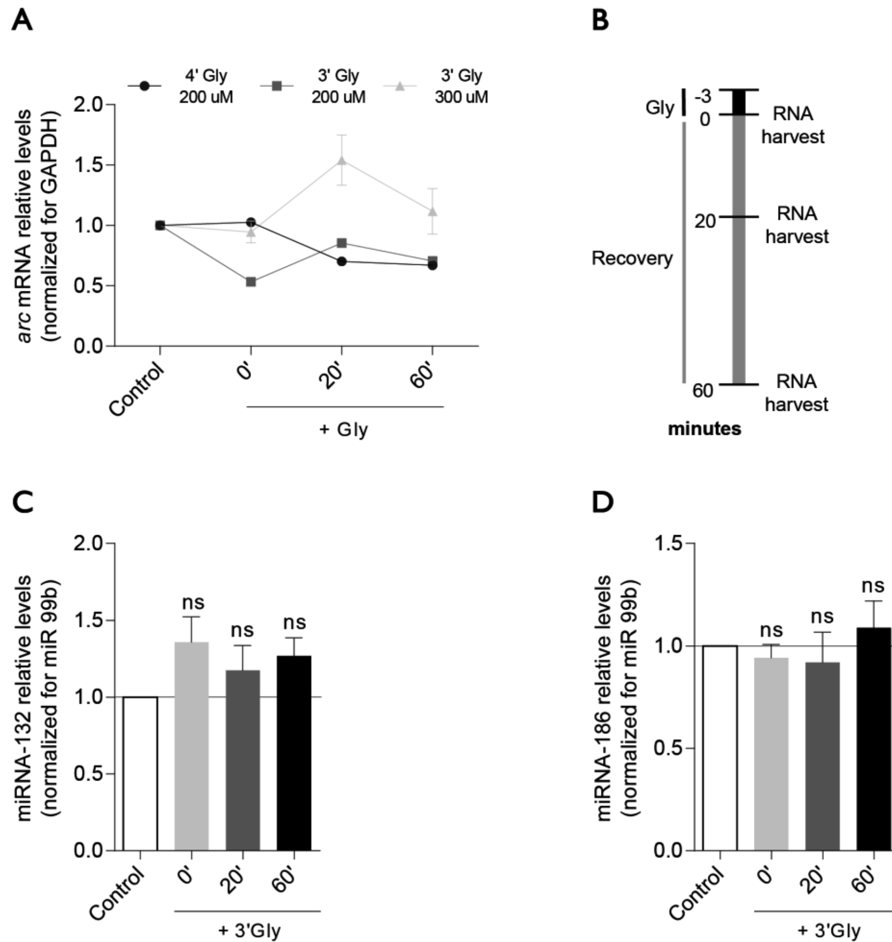

Figure 20| cLTP stimulus tends to upregulate miRNA-132 expression in 21DIV rat hippocampal cultures. Arc mRNA and miRNA relative levels upon 3 min of incubation with or without glycine (300 µM) in Mg2+-free medium, after 0, 20 or 60 min of recovery in conditioned neurobasal medium. **A.** Comparison between different tested protocols on Arc mRNA expression, where 300 µM for 3 min were the optimal condition of incubation with glycine to induce an increase of Arc mRNA levels. (200 µM, 4 min; N=1); (200 µM, 3 min; N=1); (300 µM, 3 min; N=6). **B.** Schematic representation of methodology used to induce cLTP in 21 DIV hippocampal cultures. **C.** LTP induction mediated an increased expression of miRNA-132 during 1 h (N=6) **D.** miRNA-186 levels did not change when LTP is induced (N=6). Results are presented as means ± S.E.M., when compared to control and normalized to the house-keeping gene GAPDH (**A**) and miRNA-99b (**C-D**). Statistical analysis was determined by Kruskal-Wallis test.

miRNA-186 is negatively regulated in long-term depression dependent on group I mGluR activation

Long-term depression is another paradigm of synaptic plasticity extensively studied *in vitro,* where activation of NMDARs or mGluRs induces depression of synaptic strength. These protocols are associated with different signalling pathways and, therefore, have different characteristics. It is well described that NMDAR activation induces LTD in a manner dependent on transcription, whereas mGluR activation induces LTD in a manner dependent on local protein synthesis (Bassani et al. 2013; Beattie et al. 2000; Lu and Malenka 2012). To assess whether miRNA-132 and miRNA-186 are involved in LTD and possibly unveil a mechanism associated with this regulation, both LTD induction protocols were used in 16 DIV hippocampal neurons.

Regarding mGluR-induced LTD, protocols based on incubation with DHPG, a group 1 mGluR agonist, were tested and validated by analysing Arc mRNA levels, through q-PCR (Figure 21.A). Arc, an early gene, is increased after LTD induction (either by NMDAR or DHPG activation), promoting AMPARs endocytosis (Jakkamsetti et al. 2013; Park et al. 2008; Shepherd and Bear 2011)**.** Therefore, several drug concentrations (50 µM, 100 µM) and incubation times (5, 10, 30 min) with DHPG were tested, and neurons recovered for 20 and 60 min prior RNA extraction. The results showed that hippocampal neurons incubation with 100 µM DHPG for 5 min was the most effective protocol to increase Arc expression (1.72±0.21 fold-change relatively to control) and, therefore, induce LTD dependent on mGluR activation (Figure 21.A; Figure 21.B). Hence, the effect of LTD mediated by mGluRs on hippocampal neurons in miRNA-132 and miRNA-186 was evaluated by qPCR. No effects on the expression of miRNA-132 were observed upon mGluR-mediated LTD (Figure 21.C), whereas miRNA-186 levels were decreased 20 min after induction of LTD (0.61±0.6 of control), and were still lower than control levels 1h after LTD induction (0.65±0.14 of control) (Figure 21.D).

Regarding LTD mediated by NMDA receptors, 16 DIV hippocampal dense cultures were subjected to incubation with NMDA (20 µM, 3 min) followed by a period of cell recovery of 20 or 60 min and RNA extraction (Lee et al. 2014) (Figure 22.B). Prior to miRNA analysis, this protocol was first validated by imunocytochemistry analysis of surface GluA1 in 16 DIV hippocampal cultures with low density transfected with inhibitor scramble plasmid (Figure 22.A), and Arc mRNA levels analysis by qPCR (Figure 22.C). The NMDAR-dependent LTD protocol that we used led to a decrease in area (0.23±0.06 relatively to control after 20 min and 0.31±0.07 relatively to control after 1h of stimulus), number (0.42±0.08 relatively to control after 20 min and 0.60±0.09 relatively to control after 1h of stimulus) and intensity (0.21±0.5 relatively to control after 20 min and 0.28±0.08 relatively to control after 1h of stimulus) of surface GluA1 clusters co-localized with VGluT1 (Figure 22.A). Additionally, upon NMDAR-LTD there was a raise on Arc expression (1.99±0.15 to control after 20 min and 1.91±0.15 relatively to control after 1h of stimulus) (Figure

22.C), demonstrating marked LTD induction generated by the protocol. However, the levels of neither of the miRNAs that we tested – miRNA-132 and -186 were changed upon induction of NMDAR-LTD. Indeed, miRNA-132 levels remained on basal levels under NMDA-dependent LTD (0.99±0.07 relatively to control after 20 min and 0.89±0.05 relatively to control after 1h of stimulus) (Figure 22.D) and so did miRNA-186 levels (1.08±0.09 relatively to control after 20 min and 1.02±0.17 relatively to control after 1h of stimulus) (Figure 22.E).

Therefore, miRNA-186 levels were only altered upon group 1 mGluR stimulation, a protocol dependent on protein synthesis, which may indicate a regulatory role for miRNA-186 in mechanisms dependent on protein synthesis for LTD induction. Moreover, this effect seems to be specific for miRNA-186, since miRNA-132 expression was not altered either by NMDAR or group I mGluR activation.

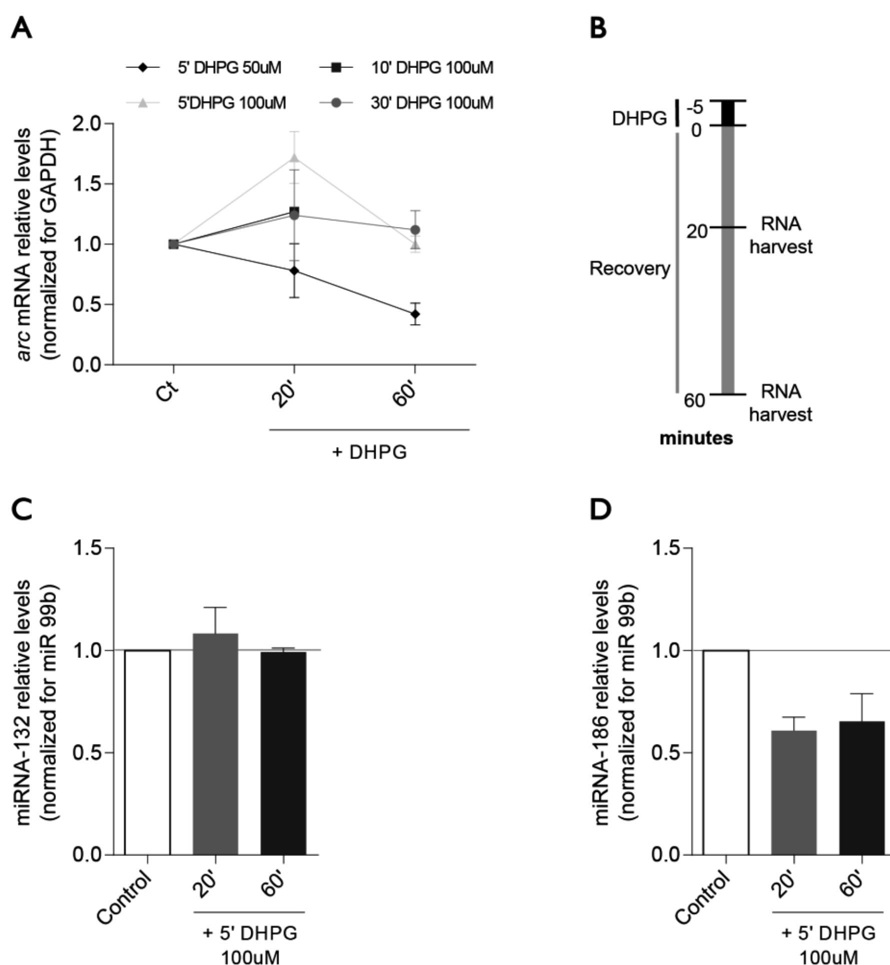

Figure 21| miRNA-186 expression is decreased upon induction of LTD dependent on group I mGluR activation. miRNA levels upon LTD induction with DHPG (100 µM, 5 min) in 16 DIV hippocampal cultures; the protocol was validated by looking at the expression of Arc mRNA **A.** Comparison between the effects of different times of incubation and DHPG concentrations on Arc mRNA expression revealed that incubation in 100 µM DHPG for 5 min was the optimal condition of incubation with DHPG to induce an increase of Arc mRNA levels (N=2). (50 µM, 5 min; N=2); (100 µM, 30 min; N=3); (100 µM, 10 min; N=1). **B.** Schematic representation of the experimental outline used to induce LTD dependent on mGluR activation with DHPG in 16 DIV hippocampal dense cultures. **C.** miRNA-132 levels were not affected by group I mGluR activation (N=2) **D.** 20 min after mGluR1-dependent LTD was induced, neurons showed decreased levels of miRNA-186 in comparison to non-stimulated cells; miRNA-186 levels remained diminished 60 min post-stimulus (N=2). Results are presented as means ± S.E.M., when compared to control and normalized to the house-keeping gene GAPDH (**A**) and miRNA-99b (**C-D**). Statistical analysis was performed using the Kruskal-Wallis test.

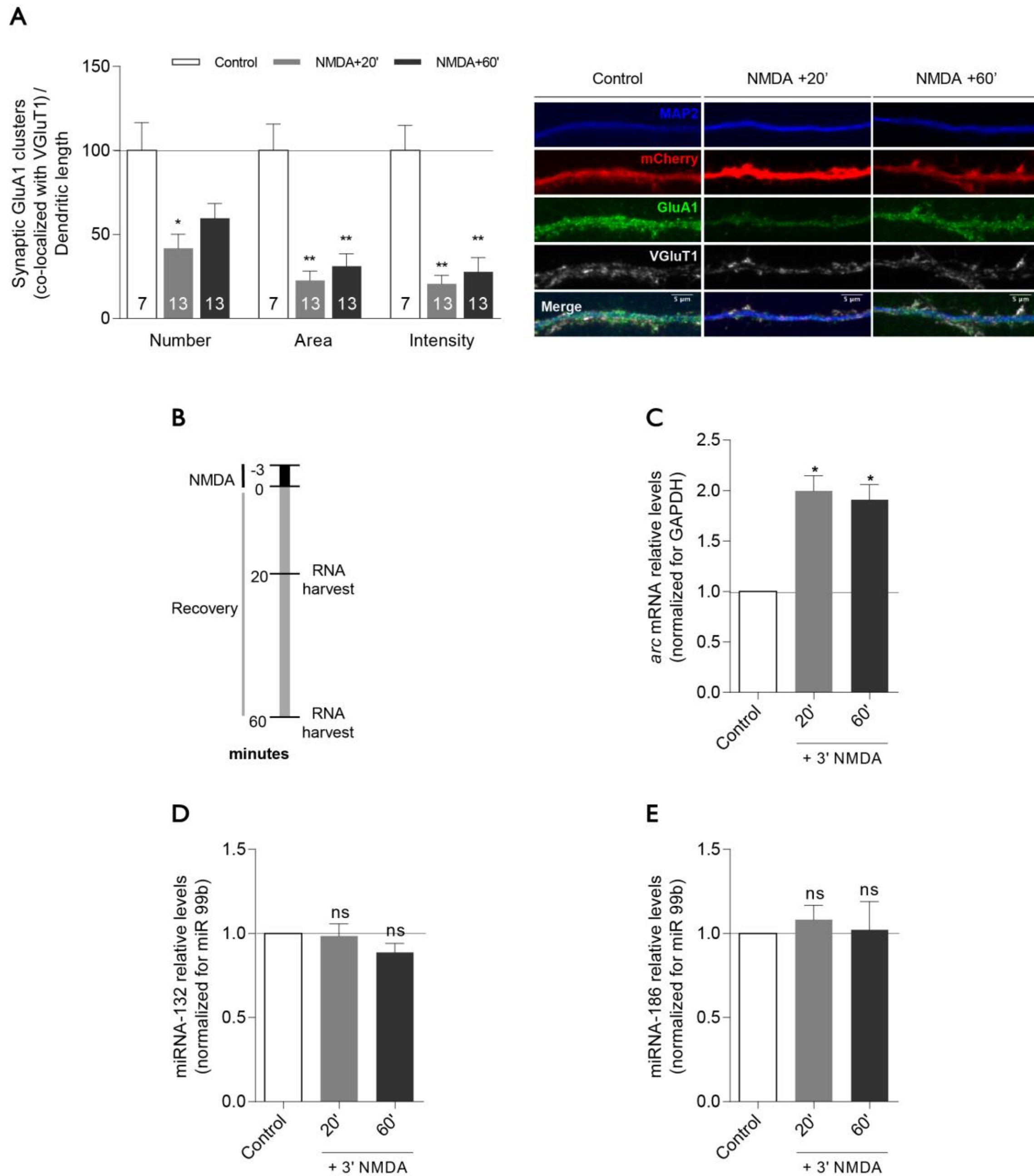

Figure 22| miRNA-132 and miRNA-186 expression levels did not change upon NMDAR-mediated LTD. Effects of NMDA treatment (20 µM, 3 min) on synaptic GluA1 clusters in 16 DIV hippocampal cultures (low density), as well as on Arc mRNA and miRNA relative levels in 16 DIV hippocampal cultures (high density). **A.** NMDA treatment of 16 DIV hippocampal Banker cultures resulted in a decrease on number, area and intensity of surface GluA1 subunit clusters co-localized with VGluT1 when compared to control non-stimulated cells. **B.** Schematic representation of treatment of 16 DIV hippocampal dense cultures to induce NMDAR-mediated LTD. **C.** After 20 and 60 min upon the NMDA stimulus, Arc mRNA levels raised 2-fold and 1.91-fold, respectively (N=4). **D** and **E.** Neither miRNA-132 (**D**) nor miRNA-186 (**E**) levels were altered by NDMAR-dependent LTD (20 µM, 3 min) either after 20 min or 60 min of cell recovery (N=4). Results are presented as means ± S.E.M., when compared to control and normalized to the house-keeping gene GAPDH (**C**) and miRNA-99b (**D-E**). GluA1 clusters measurements were performed in a single experiment, and values obtained per cell were normalized against the control mean of the experiment. Statistical analysis was determined by Kruskal-Wallis test, *p<0.05; **p<0.01 relatively to the control condition.

Inhibition of miRNA-186 had no effects on AMPA-mediated mEPSCs

Taking into account that miRNA-186 is regulated by different manipulations of neuronal activity (such as prolonged blockade of glutamate receptor activity, as previously shown in our lab, and mGluR-LTD, as shown in this work), that GluA2 is a target of miRNA-186, and that miRNA-186 was recently described to be altered in conditions such as addiction and neurodegenerative diseases (Babenko et al. 2012; Bekdash and Harrison 2015; Du et al. 2016), it was our aim to examine the role of miRNA-186 in regulating synaptic function. Therefore, we performed loss of function studies by transfecting hippocampal neurons in culture with a plasmid resulting in the production of miRNA-186 inhibitor, which blocks miRNA-186 regulation of target genes. Constitutively expressed under U6 promoter, miRNA inhibitor clone binds specifically to miRNA-186, allowing transient and stable suppression of its effects. Upon inhibiting miRNA-186, effects on synaptic transmission were assessed by whole-cell recordings in the patch-clamp mode of mEPSCs. 15 DIV hippocampal dense cultures, transfected with either miRNA-186 inhibitor (Figure 23.A) or scrambled control inhibitor (Figure 23.B) (4 days of expression) were used for whole-cell recording for 3 min in the presence of 100 µM picrotoxin (to block $GABA_A$ receptors), 500 nM TTX (to block Na^+ channels and the generation of action potentials), 50 µM AP-V (to block NMDA receptors), to block synaptic activity and measure spontaneous AMPARs-mediated currents.

Overall, miRNA-186 downregulation resulted in no significant effects on AMPAR-mediated transmission, either postsynaptic or presynaptic, since amplitude and frequency of mEPSCs were not changed in comparison to control cells (Figure 23.C-D; Figure 23.F-G). However, a slight increase on decay tau was verified under miRNA-186 inhibition (Figure 23.E; Figure 23.H).

Together, our data revealed no changes on synaptic transmission mediated by AMPARs when miRNA-186 action was inhibited.

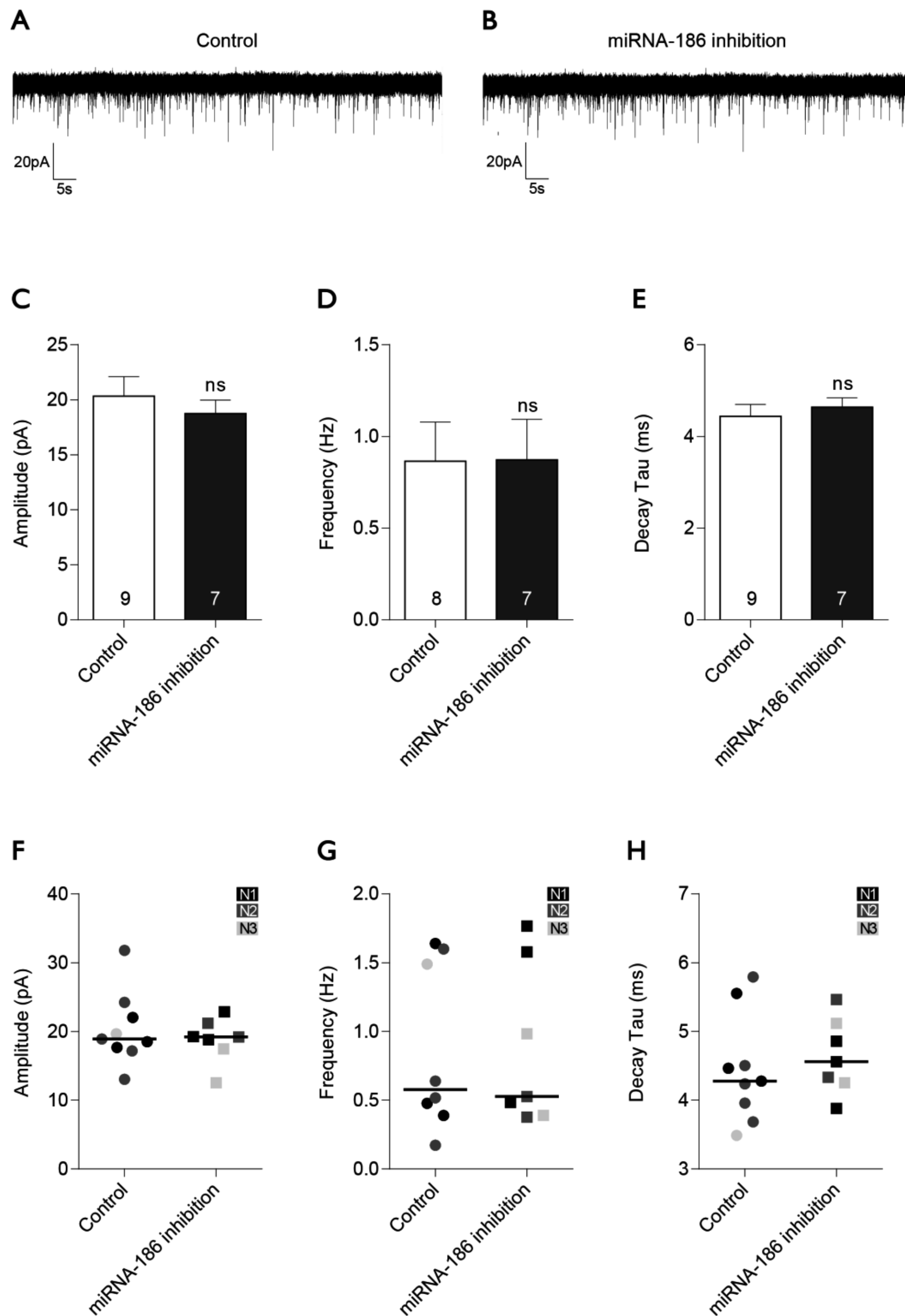

Figure 23| AMPAR-mediated mEPSCs were not changed by inhibition of miRNA-186. A-B. Representative trace of whole-cell recording during 3 min of mEPSCs mediated by AMPARs, performed in hippocampal cells transfected with scramble plasmid (**A**) or a plasmid for miRNA-186 inhibition (**B**). **C-E.** When miRNA-186 was inhibited, changes on amplitudes (**C**), frequency (**D**) and decay tau (**E**) of mEPSCs were not detected. **F-H.** Median and recording medians distributions of amplitude (**F**) and frequency (**G**) medians were not changed when miRNA-186 was inhibited. However, median of decay tau (**H**) had a tendency to increase slightly. Results are presented as means ± S.E.M. of medians (**C-E**) and as median (**F-H**), for N=3, when compared to control cells transfected with scramble plasmid, normalized to baseline amplitude (pA). Statistical analysis was determined by Kruskal-Wallis test.

Discussion

miRNA-132 and miRNA-186 levels increase during neurodevelopment

Considering alterations on structure and molecular composition of excitatory synapses throughout development, expression levels of the key molecules are tightly regulated. As demonstrated previously, the miRNA system is known to orchestrate and to be regulated by key processed in development (Fiore, Siegel, and Schratt 2008; Kosik 2006; Petri et al. 2014). Therefore, given that miRNA-132 expression is highly regulated by neuronal activity, and experience-dependent synaptic refinement occurs in early post-natal development, we hypothesized that its expression may be developmentally regulated and, moreover, inversely correlated with GluA2 expression, one of its putative targets. In agreement with previous reports (Nudelman et al. 2010; Olde Loohuis et al. 2012; Wang, Kwon, and Tsai 2012), miRNA-132 expression in rat hippocampus is progressively increased during neurodevelopment, both *in vitro* and *in vivo,* correlating with a period of active synaptogenesis (Lohmann and Kessels 2014). In fact, this miRNA has been implicated in the maturation of excitatory synapses formed by new-born neurons (Luikart et al. 2011), coupled with its established ability to induce dendritic outgrowth, branching and spine head size enlargement (Nudelman et al. 2010). Despite the regulation of p250GAP (Olde Loohuis et al. 2012) and MeCP2 (Klein et al. 2007) by miRNA-132 along maturation, miRNA-132 expression is not inversely related to GluA2 protein levels in the hippocampus *in vivo* in the first 6 postnatal weeks, with an increase of both miRNA-132 and GluA2. Conversely to *in vivo*, an opposite expression pattern of GluA2 expression was found *in vitro* during three weeks of culture, although RNA extraction method could underlie the explanation of these controversial results. Both in cultured neurons and in the postnatal hippocampus the levels of miRNA-132 and miRNA-186 increased, showing, clearly, that the expression of both these miRNAs is increased during development.

miRNA-186, a neuron-enriched miRNA that is highly expressed in the hippocampus (in comparison with other areas of the nervous system), has been described to be downregulated with aging (J. Kim et al. 2016). In dissociated cultures, an increase in miRNA-186 levels and a decrease in GluA2 mRNA levels are observed, when comparing 28 DIV to 7 DIV cultures; the decrease in GluA2 mRNA expression is not in accordance to previous studies published (Orlandi et al. 2011; Pandey et al. 2015; Pickard et al. 2000). However, these discrepancies may be related with different protocols of cell culture or RNA extraction. Our data shows that miRNA-186 has low expression levels during the first four postnatal weeks, period of active synaptogenesis. However, between P22 and P43, its expression levels raise. It is also important to emphasise that a dilution effect may be happening since the hippocampus is comprised of various cell types and this miRNA is enriched in neurons. Notably, these results appear to refute our hypothesis that an inverse expression during neurodevelopment is occurring between miRNA-186 and GluA2, since both miRNA-186 and GluA2 protein levels increase. This indicates that this miRNAs is not a major regulator of GluA2 expression during development, but does not exclude the possibility that it still contributes to GluA2

regulation during development. In fact, it is likely that during development GluA2 levels are regulated to a large extent at the level of transcription, rather than post-transcriptionally. Moreover, it is possible that these miRNAs regulate GluA2 levels mostly in mechanisms associated with synaptic plasticity,

Hence, increased expression of miRNA-132 levels coexist with neuronal maturation process and GluA2 expression. It is curious to note that miRNA-186 levels are maintained constant during early stages of postnatal neurodevelopment, but probably as a consequence of an unknown trigger mechanism, miRNA-186 ends up being upregulated after the fourth postnatal week, a late stage of neurodevelopment. It would be important to understand the significance of this upsurge in miRNA-186 levels at this developmental stage.

miRNA-132 is regulated by KCl and cLTP paradigms of neuronal activity

Over the years, it has been demonstrated that neuronal activity manipulation regulates miRNA levels, a process that fine-tunes gene expression in response to synaptic plasticity and memory formation. Hence, several studies have demonstrated that miRNA-132 expression is regulated by neuronal activity, being modulated with different paradigms of activity and in different brain regions (Sim, Bakes, and Kaang 2014).

LTP is one of the most studied paradigms of activity, and miRNAs have been shown to be altered during LTP, including miRNA-24, miRNA-34a, miRNA-132, miRNA-212 (Sim, Bakes, and Kaang 2014). Nonetheless, studies have shown that the protocols used to induce LTP and the time points selected for analysis have great impact on miRNA identified, hinting for a tight network of miRNA regulation after LTP induction. Moreover, conventional electrophysiological LTP induction protocols involve an intensive but transient activation of a small set of synapses, whereas continuous chemical networks activation lead to high proportion of potentiated synapses, being the last recommended to detect molecular and cellular changes associated with LTP (Molnár 2011). For this study, 21 DIV hippocampal neurons were submitted to a chemical LTP protocol using glycine, which was validated by verification of Arc upregulation, since this is a feature of LTP consolidation (Bramham et al. 2010; Shepherd and Bear 2011; Shepherd et al. 2006). Notably, among the three protocols tested, only treatment for 3 min with 300 µM of glycine freshly prepared, without previous NMDARs blockade, raised Arc levels consistently within 1 h after LTP induction, as previously demonstrated (Messaoudi et al. 2007; Shepherd and Bear 2011; Shepherd et al. 2006).

Moreover, miRNA-132 was positively regulated by LTP induction with glycine, which is in accordance with the literature (Eacker et al. 2011; Pai et al. 2014; Wibrand et al. 2010), although results were not statistically significant. Interestingly, this model claims that entry of calcium

through activated NMDARs (by glycine, in the absence of Mg^{2+}, in this case), triggering MEK-ERK, CaMKKIIγ and CaMKK pathways, which ultimately increase miRNA-132 transcription promoted by CREB activation. At the end, miRNA-132, by targeting p250GAP, a protein responsible for actin polymerization inhibition, promotes actin polymerization and, consequently, spine enlargement, one of the main and unique features of LTP (Magill et al., 2010; Schratt, 2009; Wayman et al., 2008, Vo et al 2005). Moreover, its effects on synaptic function could explain the augmented levels under LTP. Like BDNF (Kawashima et al. 2010), also direct NMDARs activation may upregulates AMPAR and NMDAR subunits to potentiate the synapse with increases on mEPSC amplitude and frequency. Also important to realize, our results demonstrate an immediate upregulation on miRNA-132 after glycine treatment, possibly due to an increase of pre-miRNA processing in response to synaptic plasticity (Lugli et al. 2005), leading us to speculate a role for this miRNA on LTP induction. Nevertheless, levels remained increased until up to 1 hour after stimulation, concluding that this miRNA may not only be involved on LTP induction but it could also have a function on protein synthesis-dependent mechanisms for long-lasting alteration on synaptic functions and structure that underlies LTP. In fact, miRNA-132 has already been reported to regulate glutamate receptors in the synapse (Kawashima et al. 2010), although all the exact mechanism associated with this function and its targets are still unknown. MiRNA-132 is upregulated during early and late phases of LTP by NMDARs activation, contributing to molecular and structural synaptic strength alterations.

Inversely to LTP, miRNA-132 levels were not altered neither by NMDA- nor mGluR-mediated LTD, although a slight increase has been reported in the pyramidal neurons of the hippocampus subjected to DHPG treatment (Lusardi et al. 2012) . We could speculate that these negative results derived from non-effective LTD induction, however both protocols (NMDA and DHPG treatment) applied to cells resulted on increased Arc expression, a well-known feature of LTD, caused by NMDARs and mGluRs activation to orchestrate AMPAR endocytosis (Jakkamsetti et al. 2013; Park et al. 2008; Shepherd and Bear 2011). In the same way, NMDA application yielded robust decrease on number, area and intensity of surface GluA1 clusters present at synapse of transfected hippocampal neurons, confirming, thus, LTD induction.

Alongside mechanism of synapse strength, miRNA-132 precursor and mature forms have been demonstrated to be increased when $GABA_A$ inhibitory tone is blocked by bicuculline, in a CREB-dependent manner (Wayman et al. 2008). In addition, the same authors also reported that KCl treatment increases miRNA-132 transcription, suggesting that different paradigms of neuronal activity stimulation induce miRNA-132 increase. In agreement with this data, hippocampal neurons at 16 DIV were incubated, for different time periods, with KCl. Subsequently, in order to validate KCl-depolarization paradigm, Arc expression was measured as a positive control, since Arc transcription is stimulated by membrane depolarization with KCl (Lam et al. 2009; Waltereit et al.

2001; Zheng, Luo, and Wang 2009). As expected, Arc mRNA levels increased under induced depolarization, however decreasing to basal levels overtime, leading us to validate KCl-induced depolarization. In fact, our results show a tendency for an increase of miR-132 levels. Albeit previous study reported a marked augment upon 1 h of KCl incubation (Wayman et al. 2008), KCl led to an increased miRNA-132 expression only after 2 h of KCl incubation, but slightly declining its levels at 6 h of stimulation. Nevertheless limited number of experiments did not allowed us to have a robust outcome, even though all experiments displayed the same clear tendency of increased levels of miRNA-132 under sustained enhanced neuronal activity. The mechanism behind miRNA-132 upregulation is probably the calcium influx through L-type Ca^{2+} channels that activates CaM kinase (Macías et al. 2001), BDNF synthesis with consequent ERK activation (Maharana, Sharma, and Sharma 2013). Together, CaM kinase and MEK–ERK activated pathways culminate on CREB activation, and miRNA-132 transcription lying here the explanation for miR-132 upregulation under sustained activity (Elramah, Landry, and Favereaux 2014).

Overall, our data indicate that miRNA-132 could have a role in LTP as well as in depolarization-induced sustained activation for short periods of time. Nonetheless, neither NDMAR- nor mGluR-dependent LTD had any repercussions on miRNA-132 levels. Although the exact targets and common pathway to LTP and KCl depolarization are still an enigma, indeed, miRNA-132 is an activity-dependent microRNA. Tightly regulated throughout development, this miRNA likely contributes to critical synaptic refinements underlying neuronal maturation and synaptic plasticity.

mGluR-dependent LTD regulates miRNA-186 levels.

Learning-related adaptations, comprising LTD and LTP, require neural and molecular networks to detect correlations between events, strengthening or weakening synaptic inputs, ending up reinforcing useful pathways to store information (Malenka and Bear 2004; Turrigiano 2008). Since AMPARs carry the majority of excitatory synaptic current in the central nervous system, it is not surprising that a straightforward way to regulate glutamatergic synaptic strength is to change the accumulation of glutamate receptors in the postsynaptic membrane (Malenka and Bear 2004). Given that, activity-regulated miRNA-186 regulates GluA2 expression (unpublished data from our group), we hypothesize a role for this miRNA as a regulator of synaptic plasticity mechanisms.

Since previous results from our laboratory already showed a downregulation of miRNA-186 levels by neuronal activity blockade, it was interesting to study whether this miRNA was altered in other paradigms of activity. Our experiments identified LTD induced by mGluR activation as the only paradigm among those tested with a clear tendency to regulate miRNA-186 expression levels.

After LTP induction or during maintenance of L-LTP, no effects on miRNA-186 levels were

observed, even though this mechanism is characterized by protein synthesis activation and increase in GluA2 levels. Moreover, NMDAR-mediated LTD similarly had no impact on miRNA-186 expression. Given that protein synthesis is not activated under NMDA treatment in the LTD protocol, a more marginal role for the miRNA system on synaptic depression induced by NMDAR-LTD is anticipated. Consequently, it became mandatory to analyse the impact of protein synthesis during LTD on miRNA-186 expression levels, by performing DHPG-induced LTD. Interestingly, despite the limited number of experiments, miRNA-186 levels are tendentiously decreased 20 and 60 min upon group 1 mGluRs activation, which may indicate that the regulation of miRNA-186 could be required not only on early phase of LTD, but also during the consolidation phase. Since miRNA-186 levels decreased after a short period of time, it lead us to believe that destabilization and degradation of miRNA may take place to decrease its levels, instead or concomitantly with decreased transcription, which could be also occurring and have an impact at later time-points. Albeit not directly related, in work performed on our lab, XRN2, a protein involved on miRNA degradation, was found to be upregulated under activity blockade, inversely correlating with miRNA-186 levels in the same conditions, which can indicate a signalling pathway targeting miRNA-186 for degradation. However, decreased miRNA-186 levels observed could be connected with an increase of GluA2, counteracting the key feature of LTD – synaptic GluA2 decreased levels. However, it was previously reported that DHPG treatment enhances local synthesis of GluA2 (Ju et al. 2004), which correlates nicely with decreased miRNA-186 levels upon DHPG treatment.

Considering all these results, the hypothesis that miRNA-186 could regulate synaptic function has arisen. Taking advantage of whole-cell patch-clamp, we addressed this question by using miRNA-186 plasmid construct inhibitor that efficiently blocks endogenous miRNA-186 function, in hippocampal neurons. However, neurons transfected with miRNA-186 inhibitor showed no significant alterations either on amplitude, frequency or decay tau of AMPAR-mediated mEPSCs. Indeed, taking into account the decreased intensity and area of PSD95 clusters detected upon miRNA-186 inhibition (unpublished data from Mariline Silva), the frequency of mEPSCs was expected to decrease. Whereas, the increase in the number of GluA2-containg synapses (observed by immunofluorescence studies on our lab) indicated that amplitude might be increased. However, both amplitude and frequency appear to be unaffected. One possibility is that miRNA-186 inhibition leads to the accumulation of extrasynaptic GluA2-containing AMPARs, detected by immunocytochemistry methods but that do not contribute to synaptic function under basal conditions. It is possible that miRNA-186 inhibition effects on AMPARs function are more visible under neuronal activity manipulation, such as LTD or neuronal activity blockade (paradigms that downregulate miRNA-186) and that miRNA-186 inhibition has no effects on amplitudes of AMPARs-mediated mEPSCs at basal levels. In addition, decay tau of mEPSCs was measured and results plotted as median shown a slight increase when miRNA-186 is inhibited. These results may

be relevant, in particular since decay tau alterations have already been related to different AMPAR subunit composition, specifically since an increase of GluA2-lacking receptors results on decreased decay tau (Briand et al. 2014; Jonas 2000). Accordingly, an increase on synaptic GluA2-containing AMPARs is expected to culminate in slower decay kinetics of mEPSCs. However, more recordings must be acquired to obtain robust results in order to corroborate or discard some of the hypotheses here proposed.

In summary, this work shows that miRNA-186 levels increase during late phases of neurodevelopment, although its levels do not negatively correlate with GluA2, one of its targets. Moreover, miRNA-186 levels decrease in response to group 1 mGluR-dependent LTD (but not after NMDAR-dependent LTD), indicating a possible role for this miRNA in LTD, induced by mechanism dependent of protein synthesis. Nevertheless, synaptic function is not altered by miRNA-186 inhibition at basal levels of synaptic activity, regarding frequency or amplitude of mEPSCs. However, the tendency to have an increase in the decay time as a consequence of miRNA-186 inhibition may indicate altered subunit composition of AMPAR present at active synapses.

Conclusion and future perspectives

Together these results demonstrate that both miRNA-132 and miRNA-186 are upregulated, throughout neurodevelopment, peaking at late stages of maturation, alongside with an increase in GluA2 protein levels. In fact, no inverse correlation between their expression levels indicates that regulation of GluA2 by both miRNA, although tightly regulated and contributed to critical synaptic refinements, may not be the major determinant of GluA2 expression during development, and that it may rather play a role in synaptic plasticity mechanisms.

By manipulating synaptic activity, we found that miRNA-132 and miRNA-186 are differently regulated in hippocampal neurons. Accordingly, miRNA-132 seems to have a clear tendency to be upregulated both in cLTP, due to a CREB-dependent mechanism; and in induced neuronal depolarization, although the mechanism behind this is still unknown. On the contrary, miRNA-186 responds markedly to group 1 mGluR-dependent LTD and activity blockade, by being negatively regulated, although the mechanisms behind this downregulation are unclear. However, it remains to unveil which are the activated pathways common to different stimulus paradigms that, in fact, contribute to modulate these miRNA levels, either by interfering with their expression or by affecting their degradation.

Thus, it is important to further understand how these miRNAs levels are modulated and how they regulate their targets in conditions of neuronal activation or blockade. Since miRNA-186 function is not as studied as miRNA-132, and that we have found that miRNA-186 targets GluA2 and regulates the number of excitatory and inhibitory synapses in hippocampal neurons (unpublished data), our future work will be more focused on miRNA-186. To note, prior to any new approach, the number of experiments described here will be increased to improve the robustness of these results. Posteriorly, it could be very interesting to further study LTD-induced by mGluRs activation, by manipulating miRNA-186 levels, in order to either counteract or mimic the miRNA tendency showed in our results. This will clarify the involvement of miRNA-186 in LTD-induction mechanisms, possibly by blocking or occluding LTD.

Regarding the role of miRNA-186 in regulating synaptic function, imaging experiments revealed an increase on GluA2 levels upon miRNA-186 inhibition (unpublished data), but surprisingly no alteration on postsynaptic currents was detected. One possibility is that miRNA-186 inhibition triggers the synthesis and incorporation of extrasynaptic GluA2-containing AMPARs, which do not contribute to synaptic mEPSCs under basal conditions. To test this hypothesis we will record synaptic and extrasynaptic mEPSCs when kainate is applied to cultured neurons. The outcome of these experiments should elucidate whether the increase on surface GluA2-containing AMPARs, showed in our imaging studies (unpublished data), derives from the insertion of GluA2-containing AMPARs into extrasynaptic sites.

In addition, it is important to test whether upon induction of synaptic plasticity the miRNA-186 regulation of GluA2 becomes relevant in the regulation of synaptic AMPAR currents. Therefore, mEPSCs recordings will be performed after prolonged activity blockade with Gyki and MK801, to induce synaptic scaling of AMPAR-mediated currents and a decrease in miRNA-186. We will evaluate whether Gyki and MK801-triggered synaptic scaling is perturbed when miRNA-186 levels are either decreased or increased. On the other hand, given the effect of mGluR-dependent LTD on miRNA-186 levels, we will test whether overexpression or inhibition of miRNA-186 affects mGluR-dependent LTD in hippocampal slices.

Overall this group of experiments should elucidate the role of miRNA-186 in regulating synaptic function in basal conditions and during synaptic plasticity

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