Universidad Miguel Hernández de Elche

Instituto de Neurociencias

Departamento de Neurobiología Molecular

TESIS DOCTORAL

Regulación de la plasticidad y de la capacidad de respuesta de las neuronas por el sistema de microRNAs

Regulation of neuronal plasticity and responsiveness by the miRNA system

Anna Fiorenza

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Director de Tesis:

Dr. Ángel Barco Guerrero

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Juan Lerma
Director

direccion.in@umhc.es
director.in@umh.es
www.ina.umh.es

Tel: +34 965-919221(d)
+34 965-919220 (s)
Fax: +34 965 919561

Av Ramón y Cajal s/n
Campus de San Juan
03550 SAN JUAN DE ALICANTE– ESPAÑA

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Fdo. Ángel Barco Guerrero

INSTITUTO DE NEUROCIENCIAS CSIC

abarco@umh.es
www.ina.umh.es

Tel.: +34 965 919232

Av Ramón y Cajal s/n
Campus de San Juan
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A mis padres Rita y Carmine,

por creer en mí siempre.

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And once the storm is over, you won't remember how you made it through, how you managed to survive. You won't even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won't be the same person who walked in. That's what this storm's all about.

Haruki Murakami

If you have never been called a defiant, incorrigible, impossible woman… have faith… there is yet time… Clarissa Pinkola Estes

Sono vivo e ho sorpreso nell'alba le stelle. Cesare Pavese

According to the laws of aerodynamics, the bumblebee can't fly either, but the bumblebee doesn't know anything about the laws of aerodynamics, so it goes ahead and flies anyway. Igor Sikorsky

Logic will get you from A to Z, but imagination will get you everywhere. Albert Einstein

Non lasciarti tentare dai campioni dell'infelicitá, la mutria cretina, della serietá ignorante. Sii allegro. Ti insegnano a non splendere. E tu splendi, invece. Pier Paolo Pasolini

C'est finalement au plus fort de l'hiver, que j'ai compris qu'il existait en moi un invincible printemps. Albert Camus

Foolery, sir, does walk about the orb, like the sun; it shines everywhere! William Shakespeare

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ABSTRACT

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Regulation of neuronal plasticity and responsiveness by the miRNA system

MicroRNAs (miRNAs) are small regulatory RNAs that contribute to fine-tuning regulation of gene expression by mRNA destabilization and/or translational repression. Many approaches have been used to clarify the role of individual miRNAs but, recently, a new hypothesis about their mechanism of action proposes the importance to consider miRNA system as a whole. The ablation of the RNase Dicer, essential for the maturation of most miRNAs, is a useful model to study the effect of the loss of miRNA system in different tissue and cellular types. We used a conditional and inducible mouse model for Dicer ablation in order to investigate the role of miRNAs in adult glutamatergic neurons of the forebrain. To gain molecular insight into the role of Dicer and the microRNA system in hippocampal function and plasticity, we conducted two complementary RNA-seq screens for mRNAs and miRNAs to identify specific molecules and processes affected by Dicer and miRNAs loss. Functional genomics analyses predicted the main biological processes and phenotypes associated with impaired microRNA maturation, including categories related to microRNA biology, signal transduction, seizures, and synaptic transmission and plasticity. Consistent with these predictions, we found that, soon after recombination, Dicer-deficient mice exhibited an exaggerated seizure response, enhanced induction of immediate early genes in response to novelty and kainate administration, stronger and more stable fear memory, obesity associated to hyperphagia and increased excitability of CA1 pyramidal neurons. In the long term, we also observed slow and progressive excitotoxic neurodegeneration. Overall, our results indicate that interfering with microRNA biogenesis causes an increase in neuronal responsiveness and disrupts homeostatic mechanisms that protect the neuron against over-activation, which may explain both the initial and late phenotypes associated with the loss of Dicer in excitatory neurons. Taken together, our results show that miRNA system may play a critical role in setting a threshold for neuronal activation acting like a molecular brake for important activity-induced processes, such as memory and feeding behavior, and protecting sensitive neurons against over-activation.

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Regulación de la plasticidad y de la capacidad de respuesta de las neuronas por el sistema de microRNAs

Los microRNAs (miRNAs) son pequeños ARN no codificantes capaces de regular de forma sutil la expresión génica a través de la desestabilización de los ARN mensajeros y/o la represión de la síntesis proteica. Numerosos estudios han contribuido a aclarar el papel de miRNAs individuales. Sin embargo, recientemente, nuevos modelos para explicar su mecanismo de acción han resaltado la importancia de considerar el sistema de miRNA en su conjunto. La ablación de la RNasa Dicer, esencial para la maduración de la mayoría de los miRNAs, representa un modelo útil para estudiar el efecto de la pérdida del sistema de miRNAs en diferentes tejidos y tipos celulares. En este trabajo, utilizamos una cepa de ratón que permite la ablación de Dicer de forma condicional e inducible con el fin de investigar el papel de los miRNAs en las neuronas glutamatérgicas del cerebro anterior en animales adultos. Para obtener una perspectiva molecular en el papel de Dicer y del sistema de microRNA en la función y plasticidad del hipocampo, realizamos dos experimentos de rastreo genómico complementarios usando la técnica de RNA-Seq, explorando respectivamente mRNAs y miRNAs, para identificar moléculas y procesos específicamente afectados por la pérdida de Dicer. Los resultados de estos análisis nos han permitido identificar los principales procesos y fenotipos biológicos asociados con la alteración en la maduración de los miRNAs, incluyendo categorías relacionadas con la biología de miRNAs, la transducción de señales, la epilepsia, la transmisión sináptica y la plasticidad neuronal. De forma consistente con estas predicciones, encontramos que poco después de la recombinación, los ratones deficientes para Dicer exhibían un fenotipo complejo que incluye: una respuesta exagerada a drogas pro-epilépticas, una mayor inducción de genes inmediatos tempranos en respuesta tanto a novedad como a la administración de kainato, la formación de memorias más fuertes y estables, obesidad mórbida asociada a la hiperfagia y un aumento de la excitabilidad de las neuronas piramidales de CA1. Además, a más largo plazo, también observamos una lenta y progresiva neurodegeneración. En conjunto, nuestros resultados indican que interferir con la biogénesis de miRNAs provoca un aumento en la capacidad de respuesta neuronal y altera los mecanismos homeostáticos que protegen la neurona contra el exceso de activación, lo que puede explicar tanto los fenotipos tempranos como tardíos asociados con la pérdida de Dicer en neuronas excitadoras del cerebro anterior. Además, nuestros resultados sugieren que el sistema de los miRNA puede jugar un papel crítico en el establecimiento de un umbral para la activación neuronal actuando como un freno molecular para importantes procesos

inducidos por actividad, tales como la memoria y la regulación de la toma de alimentos, protegiendo las neuronas frente a la sobre-activación.

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INDEX

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GENERAL INDEX

FIGURE INDEX

ABBREVIATIONS AND ACRONYMS

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Abbreviations and acronyms

3V	Third Ventricle
AD	Alzheimer Disease
ADF	Actin-depolymerizing Factor
AGO	Argonaute
Agrp	Agouti-related protein
AKT	RAC-alpha serine/threonine-protein kinase or Protein Kinase B (PKB)
ALMS	Alström Syndrome
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA Receptor
AP	Action Potential
Apt1	Acyl Protein Thioesterase 1
ARC	Arcuate nucleus
Arc	Activity-Regulated Cytoskeleton-associated protein
Armi	Armitage
Arpc3	Actin-related Protein 2/3 Complex subunit 3
BBB	Blood-Brain Barrier
BDNF	Brain-derived Neurotrophic Factor
Bsn	Bassoon Presynaptic Cytomatrix Protein
Cacna1g	Calcium Channel, Voltage-Dependent, T Type, Alpha 1G Subunit
CamKII	Calcium-Calmoduline Kinase II
CART	Cocaine And Amphetamine Regulated Transcript
CCK	Cholecystokinin
CCR4	Chemokine (C-C Motif) Receptor 4
CN	Cystic Nephroma
CNOT	CCR4-NOT Transcription Complex
CNS	Central Nervous System
CR	Calorie Restriction
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
CRH	Corticotropin Releasing Hormone
DAPI	4',6-diamidino-2-phenylindole
DAT	Dopamine Active Transporter
DDH motif	Aspartate-Aspartate-Histidine box
DDRNA	DNA-Damage-induced small RNAs
DDX3X	DEAD (Asp-Glu-Ala-Asp) Box Helicase 3, X-Linked
DE	Differentially Expressed
DEAH	Aspartate-Glutamate-Alanine-Histidine box
DEXH	Aspartate-Glutamate-X-Histidine box
DG	Dentate Gyrus
DGCR8	DiGeorge Syndrome Critical Region Gene 8
DHHC9	DHHC (aspartate-histidine-histidine-cysteine)-type containing 9 palmitoyltransferase
diRNA	Double-stranded Break-induced RNA
DMH	Dorsomedial hypothalamic nucleus
dsRNA	Double-stranded RNA

EGR1	Early Growth Response Protein 1
EIF2C1	Eukaryotic Initiation Factor 2C1/Argonaute 1 (Ago1)
EM	Electron Microscopy
EMX	Empty Spiracles Homeobox 1
endo-shRNA	Endogenous Short-Hairpin RNAs
EPM	Elevated Plus Maze
EPSC	Excitatory Post-Synaptic Potential
ERK	Extracellular Signal-Regulated kinase
Esr1	Estrogen Receptor 1
FC	Fold Change
FC	Fear Conditioning
FD	Food Deprivation
FI	Food Intake
FMR1	Fragile X Mental Retardation 1
FMRP	Fragile X Mental Retardation Protein
FOS	FBJ Murine Osteosarcoma Viral Oncogene Homolog
GABA	γ-Aminobutyric acid
GABAR	GABA receptor
GFAP	Glial Fibrillary Acidic Protein
GHSR	Growth Hormone Secretagogue Receptor
GLP	Glucagon-like Peptide
GluR	Glutamate Receptor
GO	Gene Ontology
GWAS	Genome-Wide Association Study
H2-Q7/8/9	Histocompatibility 2, Q Region locus 7/8/9
HC	Homecage
HCN	Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel
HFD	High-Fat Diet
HTT	Huntington Disease
IEG	Immediate Early Gene
IHC	Immunohistochemistry
InsR	Insulin Receptor
IRS2	Insulin Receptor 2
JAK	Janus Kinase 2
JGCT	Juvenile granulosa cell tumor
KA	Kainate
KCNT	Potassium Channels, Sodium-Activated subfamily T
KO	Knockout
LH	Lateral Hypothalamus
LIMK1	LIM Domain Kinase 1
LNA	Locked Nucleic Acid
Loq	Loquacious
LTD	Long-Term Depression
LTP	Long-Term Potentiation
AHP	Afterhyperpolarization

MC4R	Melanocortin 4 Receptor
MCH	Melanin-Concentrating Hormone
MECP2	Methyl CpG Binding Protein 2
MEF2	Myocyte Enhancer Factor 2
miRNA	microRNA
miscRNA	Miscellaneous RNA
MNG	Multinodulal Goiter
MOV10	Moloney Leukemia Virus 10
MRE	miRNA Response Element
mRNA	Messenger RNA
MSH	Melanocyte-Stimulating Hormone
MSK	Mitogen- and Stress-activated Protein Kinase
TOR	Target of Rapamycin
NC	Neural Crest
NE	Novelty Exposure
NeuN	Neuronal Nuclei (Feminizing Locus on X-3, Fox-3, Rbfox3, or Hexaribonucleotide Binding Protein-3)
NF-kβ	Nuclear Factor kappa-Light-Chain-Enhancer of Activated B Cells
NMDA	N-Methyl-D-Aspartate
NMDAR	NMDA Receptor
Npas4	Neuronal PAS Domain Protein 4
NPY	Neuropeptide Y
NR2A	NMDA Receptor subunit 2A
ObR	Leptin Receptor
OF	Open Field
OXM	Oxyntomodulin
OXT	Oxytocin/Neurophysin
P250GAP	Rho GTPase activating protein 32 (ARHGAP32)
PACT	Protein Kinase, Interferon-Inducible Double Stranded RNA-dependent Activator (PRKRA)
PAK1	p21 Protein (Cdc42/Rac)-Activated Kinase 1
PAZ	Piwi-Argonaut-Zwille
Pcp2	Purkinje Cell Protein 2
PI3K	Phosphatidylinositol-3-Kinase
PIWI	P-Element Induced Wimpy Testis
Pol	Polimerase
POMC	Pro-Opiomelanocortin
PP	Pancreatic Polypeptide
PPB	Pleuropulmonary Blastoma
PSD	Post-Synaptic Density
PTEN	Phosphatase and Tensin Homolog
PVN	Paraventricular Hypothalamic Nucleus
PWS	Prader-Willi Syndrome
PYY	Peptide YY
RAC	Ras-Related C3 Botulinum Toxin Substrate
RBP	RNA Binding Protein
RE1	Repressor Element 1

Abbreviations and acronyms

REST	RE1-Silencing Transcription Factor/Neural-Restrictive Silencer Factor
RISC	RNA-Inducing Silencing Complex
Rnt1	RNase Three
rRNA	Ribosomal RNA
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
sAHP	slow Afterhyperpolarization
SE	Status Epilepticus
shRNA	Short-Hairpin RNA
SINE	Short Interspersed Element
siRNA	Small Interfering RNA
Sirt	Sirtuin
snoRNA	Small Nucleolar RNA
SNP	Single-Nucleotide Polymorphism
snRNA	Small Nuclear RNA
STAT	Signal Transducer And Activator Of Transcription
SV2A	Synaptic vesicle glycoprotein 2A
TLE	Temporal-Lobe Epylepsy
TMX	Tamoxifen
TNRC6C	Trinucleotide Repeat Containing 6C
TRBP	TAR (HIV-1) RNA Binding Protein
TRH	Thyrotropin-Releasing Hormone Receptor
tRNA	Transfer RNA
UTR	Untranslated Region
VMH	Ventromedial hypotalamic nucleus
WNT	Wingless
XRN1	5'-3' Exoribonuclease
Y1-5	Neuropeptide Y Receptor 1-5

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INTRODUCTION

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1. Dicer and the microRNA system

1.1 MicroRNA essentials

MicroRNAs (miRNAs) are a class of endogenous, small non-coding RNAs of approximately 21-23 nucleotides that act as post-transcriptional regulators of gene expression. More specifically, these molecules post-transcriptionally repress gene expression by base pairing to the complementary sequence in the 3'-untranslated region of target messenger RNAs (mRNAs) interfering with their translation and/or promoting their degradation (Nilsen 2007, Filipowicz et al. 2008, Carthew and Sontheimer 2009, Inui et al. 2010). The discovery of the first miRNAs took place two decades ago during the characterization of *C. elegans* genes that control the timing of larval development revealing two small regulatory RNAs, known as lin-4 and let-7 (Lee et al. 1993, Reinhart et al. 2000). MiRNAs have since been found in plants, animals, green algae and viruses (Griffiths-Jones et al. 2008) and, actually, seem to constitute about the 1-2% of genes in worms, flies and mammals (Ruby et al. 2006, Landgraf et al. 2007, Ruby et al. 2007, Bartel 2009). Other types of small RNAs have been also found in animals, plants, and fungi. These include endogenous small interfering RNAs (siRNAs) (Reinhart and Bartel 2002, Ambros et al. 2003) and Piwi-interacting RNAs (piRNAs) (Aravin et al. 2007). Like miRNAs, many of these other RNAs function as guide RNAs within the broad phenomenon known as RNA silencing, but miRNAs differ from these other classes of small RNAs in their biogenesis: miRNAs derive from transcripts that fold back on themselves to form distinctive hairpin structures (Bartel 2004) (more details below), whereas the other types of endogenous small RNAs derive either from much longer hairpins that give rise to a greater diversity of small RNAs, from bimolecular RNA duplexes (such as the siRNAs), or from precursors without any suspected double-stranded character (piRNAs).

1.2 miRNA pathways

1.2.1 Canonical pathway for miRNAs biogenesis

MiRNAs biogenesis starts with the transcription of the miRNA encoding gene by the RNA polymerase II (Pol II) leading to the generation of a primary miRNA transcript (pri-miRNA). In the nucleus, the pri-miRNA is cleaved by the nuclear ribonuclease III (RNase III) enzyme Drosha that acts in the microprocessor complex, a double-stranded RNA-binding domain protein (see section 1.3 for further detail). Drosha selectively cleaves NA hairpins that have a relatively large terminal loop (usually ~10 bp (Zeng et al. 2005, Zeng and Cullen 2006, Zhang and Zeng 2010). The cleavage product is an approximately 70-100 nucleotides precursor miRNA hairpin (pre-miRNA) that is then translocated to the cytoplasm by exportin 5, a specific nuclear export factor that binds directly pre-miRNAs, in a guanosine triphosphate (GTP) -dependent manner (Yi et al. 2003, Lund et al. 2004). Once in the cytoplasm, the pre-miRNA is processed to a ~22 nucleotides intermediate miRNA duplex by the RNase III enzyme Dicer, which is the catalytic component of a cytoplasmic cleavage complex including many accessory proteins such as TRBP, Loquacious/PACT and FMR1 (Kosik 2006, Krol et al. 2007, MacRae et al. 2007). The resulting miRNA duplex, referred to as the miR/miR* duplex, is composite of 5'-phosphorylated 2'-3'-hydroxylated RNAs that are later separated into the two RNA strands. One strand, named guide strand (also called 5p), typically with a relative lower stability of base pairing at the 5'-end according to the so called "thermodynamic asymmetry rule" (Khvorova et al. 2003, Schwarz et al. 2003), is translocated to the RNA-inducing silencing complex (RISC) responsible for silencing target mRNAs (Fuchs et al. 2004, Murchison and Hannon 2004, Sontheimer 2005). In fact, selection of the guide strand is not random, but rather asymmetric, and depends mainly on the thermodynamic stability of the ends of the duplex. The strand with the less stable 5′-end is more likely to be selected as guide strand whereas the other strand, with a more stable 5′-end, serves as the passenger strand (Khvorova et al.

2003, Schwarz et al. 2003). Nevertheless, additional strand selection mechanisms can act on miR/miR* duplex and, in many cases, both strands are likely to be functional (Okamura and Lai 2008). The 5'-phosporylated end of the mature miRNA is critical for the interaction with the Argonaute (AGO) protein and its loading in the RISC complex (Ma et al. 2005, Parker et al. 2005, Parker 2010) to form the core of the complex. The mature miRNA serves as a guide to recruit AGO and associated cofactors to target mRNAs, resulting in mRNA destabilization and translational repression (Huntzinger and Izaurralde 2011, Pasquinelli 2012).

Argonaute (AGO) proteins are the core components of the RISC complex that modulate miRNA biogenesis, but they also regulate chromosome structure and function and provide an innate immune defence against viruses and transposons through several mechanisms (Hutvagner and Simard 2008). There are three categories of Argonaute proteins including the Ago, Piwi and worm specific subfamilies (Tolia and Joshua-Tor 2007, Hock and Meister 2008). Members of the Ago subfamily function in siRNA and miRNA pathways, while the Piwi subfamily interacts with piRNAs to regulate gene expression primarily in the germ-line of animals. Argonaute proteins are present across animal and plant species. Their number and roles vary from species to species. The yeast *Schizosaccharomyces pombe* has only one Argonaute protein, while *C. elegans* has a total of 27 with specific members dedicated to miRNA (i.e. Argonaute-Like Genes 1 and 2, ALG-1/2) or RNAi (i.e. RNAi Defective 1, RDE-1) pathways. *Drosophila melanogaster* has 5 Argonautes, but miRNAs primarily load onto Ago1. The mouse and human genomes encode 8 Argonautes with Agos 1–4 forming the Ago subfamily. MiRNAs and siRNAs seem to load indiscriminately onto these four Argonautes, although Ago2 is the only catalytically active one capable of cleaving mRNAs that pair with the loaded small RNA (Finnegan and Pasquinelli 2013). The catalytic activity of Argonaute is proposed to have a dual role in RISC complex. Indeed, in addition to acting on miRNAtarget mRNAs, the presence of its endonuclease activity

in the complex enables the uptake of the mature products, freeing Dicer for more precursor substrates. In some cases, Argonaute exerts its endonucleolytic cleavage activity on miRNA precursors that contain well-paired stem regions. In mammalian cells, Ago2 has been shown to cleave the 3′ arm of the hairpin, producing an Ago2-cleaved precursor miRNA (ac-pre-miRNA) prior to Dicer processing (Diederichs and Haber 2007).

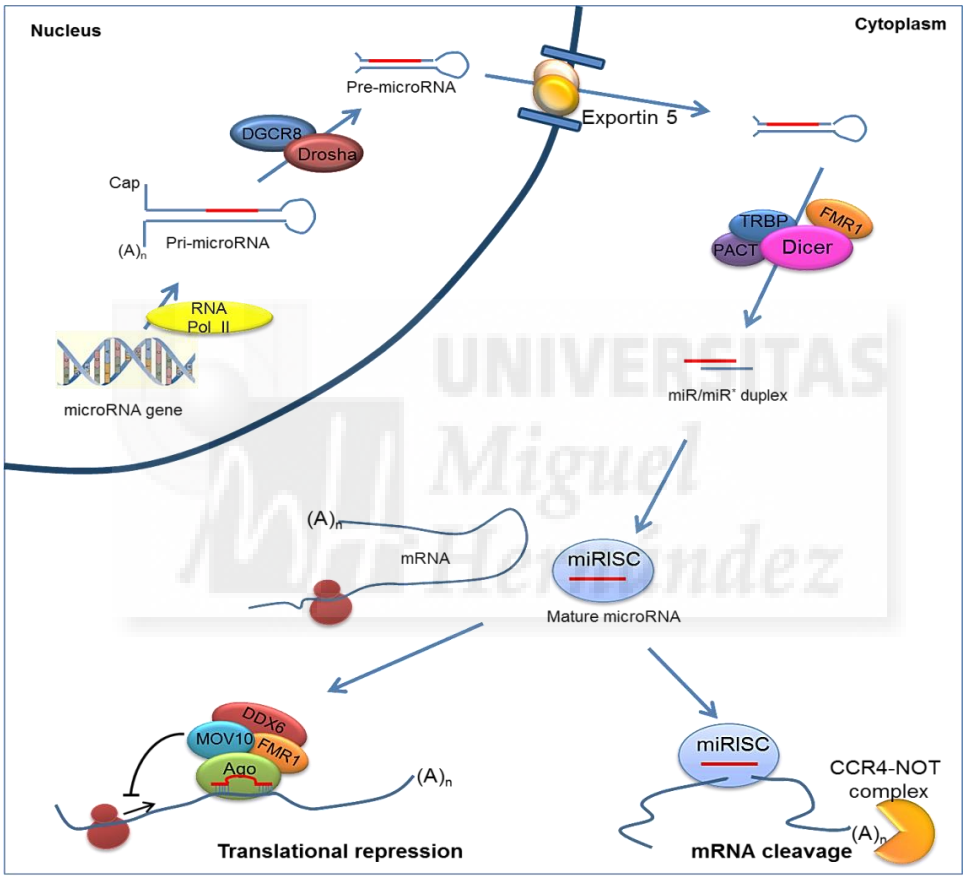

Figure 1. Canonical miRNA biogenesis pathway. Transcription of miRNA gene by the RNA polimerase II (RNA pol II) leads to the formation of a primary miRNA transcript (pri-miRNA), which is cleaved in the nucleus by the microprocessor complex, including Drosha and DGCR8, producing a precursor miRNA (pre-miRNA). The pre-miRNA hairpin is exported to the cytoplasm by exportin 5 and, there, is processed by Dicer to an intermediate miR/miR* duplex. Depending on the thermodynamic characteristics of the miRNA duplex, one strand (mature miR or miR 5p) is loaded into a multiprotein complex named miRNA-induced silencing complex (miRISC), whereas the passenger strand (or miR 3p) is usually degraded. The resulting miRNA-associated miRISC is guided to target mRNA by imperfect pairing in its 3' UTR. This interaction leads to translational repression and/or target mRNA degradation. Adapted from **Schratt 2009**.

It is hypothesized that this intermediate step may play a role in determining mature miRNA strand selection. In spite of the complementary degree between the miRNA and its target, with perfect base pairing increasing miRNA decay rates (Ameres et al. 2010, Baccarini et al. 2011), Argonaute proteins influence the levels of associated miRNAs both protecting them from degradation and acting like a limiting factor for miRNA biogenesis, given that the over-expression of any of the four Argonaute proteins in human cells leads to a global increase in mature miRNAs (Diederichs and Haber 2007). The other strand of the miRNA duplex, the passenger strand (also named miR* or 3p), is usually degraded although recently several groups demonstrated a regulatory activity for these RNAs (Jazdzewski et al. 2009, Guo and Lu 2010, Mah et al. 2010, Yang et al. 2011a, Li and Yang 2012, Chang et al. 2013, Shan et al. 2013, Winter and Diederichs 2013, Yang et al. 2013, Wu and Arora 2014). The above mentioned steps for miRNA production are also referred to as canonical biogenesis pathway (**Figure 1**).

1.2.2 Non-canonical pathway for miRNAs biogenesis

The study of miRNA has specially benefited from deep-sequencing technologies. Indeed, the length of miRNAs makes next generation sequencing instruments ideal for genome-wide discovery of miRNA gene (Mardis 2008). The sequencing of small RNAs is followed by an extensive bioinformatics-based characterization that examines the genomic locations and the potential secondary structure formation to identify putative miRNAs (Miyoshi et al. 2010). This approach has identified several classes of miRNAs that, although bypassing key steps involved in the canonical biogenesis pathway, maintain a length of ~22 nucleotides and the presence of a hairpin precursor (Berezikov et al. 2006), thereby demonstrating the ability and flexibility of cells to generate these RNAs. As summarized in **Figure 2**, Drosha- and Dicer-independent pathways for miRNAs biogenesis have been discovered and frequently referred to as non-canonical miRNA pathways (Miyoshi et al. 2010).

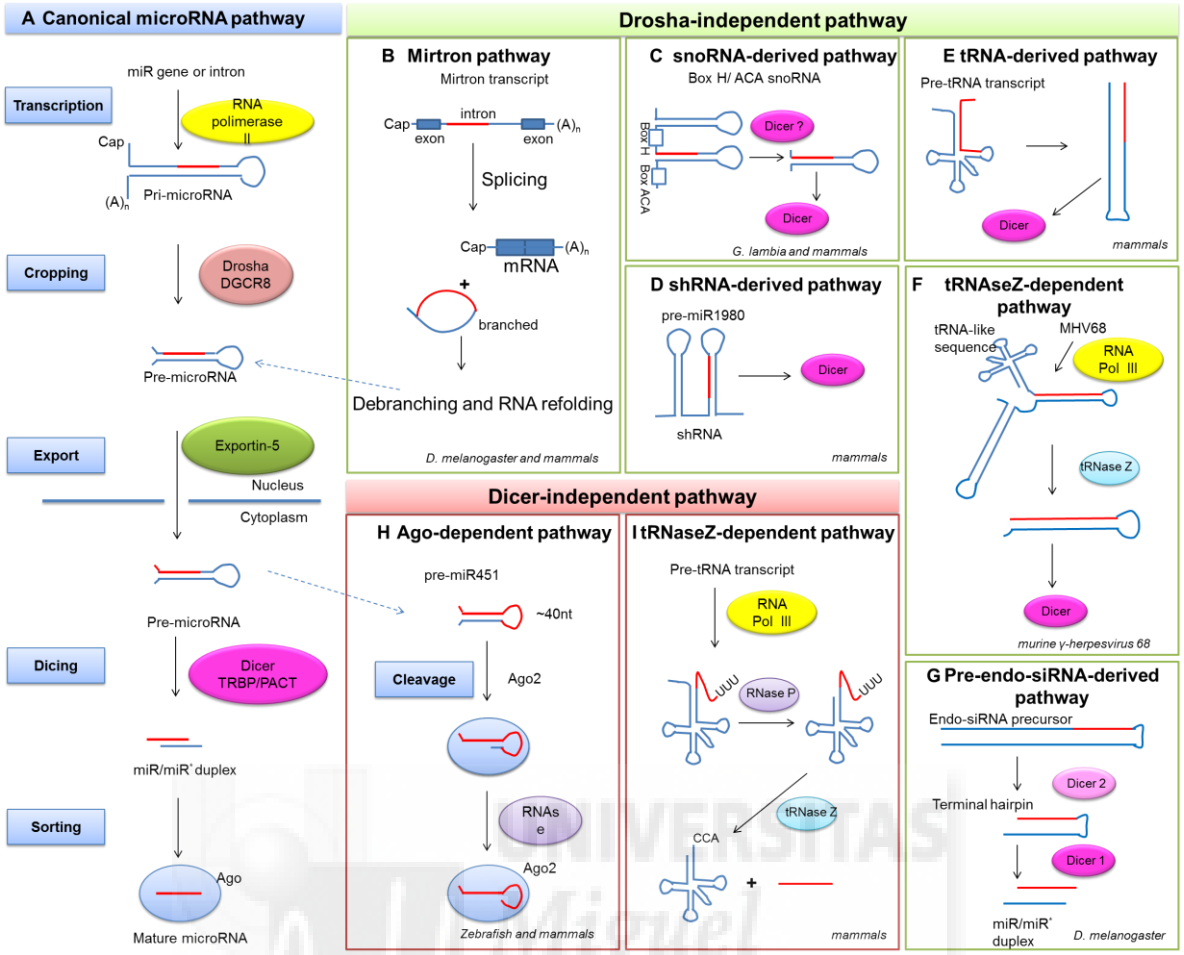

Figure 2. Canonical and non-canonical pathway for miRNA biogenesis. A. Canonical miRNA biogenesis pathway. **B-G.** Drosha-independent pathways. **B.** Mirtron-derived pathway. Short introns with hairpin potencial can be spliced and debranched into pre-miRs that bypass Drosha cleavage. Debranched mirtrons access the canonical pathway during nuclear export, then are cleaved by Dicer and loaded into miRISC. **C.** snoRNA-derived pathway. Processing of small nucleolar RNAs (snoRNAs) is Dicer-dependent but Drosha-independent. **D.** shRNA-derived pathway. Short-hairpin RNAs could be precursor of miRNAs. The predicted secondary structure of the presumed miR1980 precursor is shown. Its processing is similar to C. **E.** tRNA-derived pathway. Predicted secondary structure for the tRNA cloverleaf structure and alternative shRNA hold. **F.** tRNase Z-dependent pathway. miRNAs encoded by MHV68 derived from one arm of of two proposed pre-miR-like stem loops located 3' of the tRNA-like structure.Its nuclear processing is tRNase Z-dependent . After the nuclear export, the pre-miRNA is processed by Dicer. **G.** miRs derived from a terminal hairpin of an endo-siRNA precursor. Endo-siRNA precursors are processed by Dicer2 to yield pre-miR-like terminal hairpins, which are processed by Dicer1. **H-I.** Dicer-independent pathway. **H.** Ago-dependent pathway. Although Drosha cleavage proceeds normally, the Dicer step is skipped and the pre-miR451 is loaded directly into Ago2. **I.** tRNase Z-dependent pathway. A 3'-trailer fragment of a precursor tRNA is released by a cleavage by tRNase Z. Representative organisms that use the different pathways are indicated at the bottom of the panels. Adapted from **Miyoshi et al. 2010**.

In particular, miRNAs that derives from short introns, small nucleolar RNAs (snoRNAs), endogenous short-hairpin RNAs (endo-shRNAs), tRNAs and double stranded RNAs (dsRNAs) can bypass the Microprocessor-mediated cleavage. In the case of the Dicer-independent pathway, the key players in miRNAs production are AGO proteins. AGO proteins consist in an amino-terminal domain, the Mid domain and their signature PAZ and PIWI domains (Parker 2010). The PAZ and Mid domains help to anchor the small RNA guide, with the PAZ domain binding the 3'-end of the RNA and the Mid domain providing a binding pocket for the phosphorylated 5'-end. The PIWI domain forms an RNase H-like fold in the tertiary structure (Song et al. 2004) (**Figure 3**).

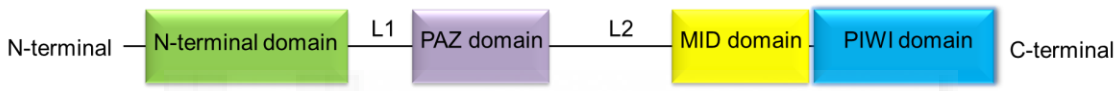

Figure 3. Human Argonaute 2 (Ago2) protein. Representative structure of human Ago2 with relative domains. More details in the text.

As previously mentioned, of four AGO proteins in mammals, only Ago2 has the endonuclease (Slicer) catalytic activity and the DDH motif that serve as a metal-coordinating site (Song et al. 2004, Miyoshi et al. 2010, Parker 2010). Due to its peculiarity, Ago2 can be also involved in miRNAs maturation. In fact, several genetic and biochemical experiments have demonstrated the existence of a Drosha-dependent and Dicer-independent pathways for miRNA-451 biogenesis for which the involvement of Ago2 is crucial (Cheloufi et al. 2010, Chong et al. 2010). Thus, the short pre-miRNA-451 generated by Drosha is directly loaded onto Ago2 and the Ago2 catalytic domain mediates a first cleavage to produce an intermediate that is cleaved by an unknown cellular nuclease to generate the mature miRNA-451. Several studies have also identified a large number of small RNAs derived from tRNAs in humans that are associated with Ago proteins (Cole et al. 2009, Haussecker et al. 2010) and produced

in a Dicer-independent and tRNase Z-dependent manner, an endoribonuclease generally responsible for the generation of the mature 3'end of tRNA molecules.

1.3 Ribonuclease III: structure and function

The enzymes belonging to the ribonuclease III (RNase III) family are defined by characteristic RNase III domains, which, as dimeric modules, confer the unique ability to cleave double-stranded RNA (dsRNA). The ability to recognize and bind dsRNA is conferred by a dsRNA binding domain (dsRBD) that is common to all RNase III. Three structural classes of RNase III have been described based upon protein complexity (**Figure 4**).

1.3.1 Class I RNase III

Class I enzymes are the simplest, consisting of those found in bacteria and simple eukaryotes, such as RNase III in *Escherichia coli* and Rnt1 in *Saccharomyces cerevisiae*. These are thought to be the antecedents of the more complex class II Drosha and class III Dicer proteins. Class I enzymes contain only one catalytic endonuclease domain and achieve the dimeric catalytic RNase module by forming dimers, whereas the more complex class II and class III members use intramolecular dimerization of their two RNase domains (Johanson et al. 2013). The ancestral RNase III members, such as bacterial RNase III, process ribosomal RNA (rRNA), whereas yeast RNase III is additionally able to process small nucleolar RNA (Chanfreau et al. 1998), small nuclear RNA (Chanfreau et al. 1997, Abou Elela and Ares 1998) and messenger RNA (mRNA) (Bardwell et al. 1989, Matsunaga et al. 1996). Until recently, it was thought that these abilities were not conserved in the more complex class II and III enzymes. Evolution of these enzymes in higher eukaryotes led to the accumulation of additional domains.

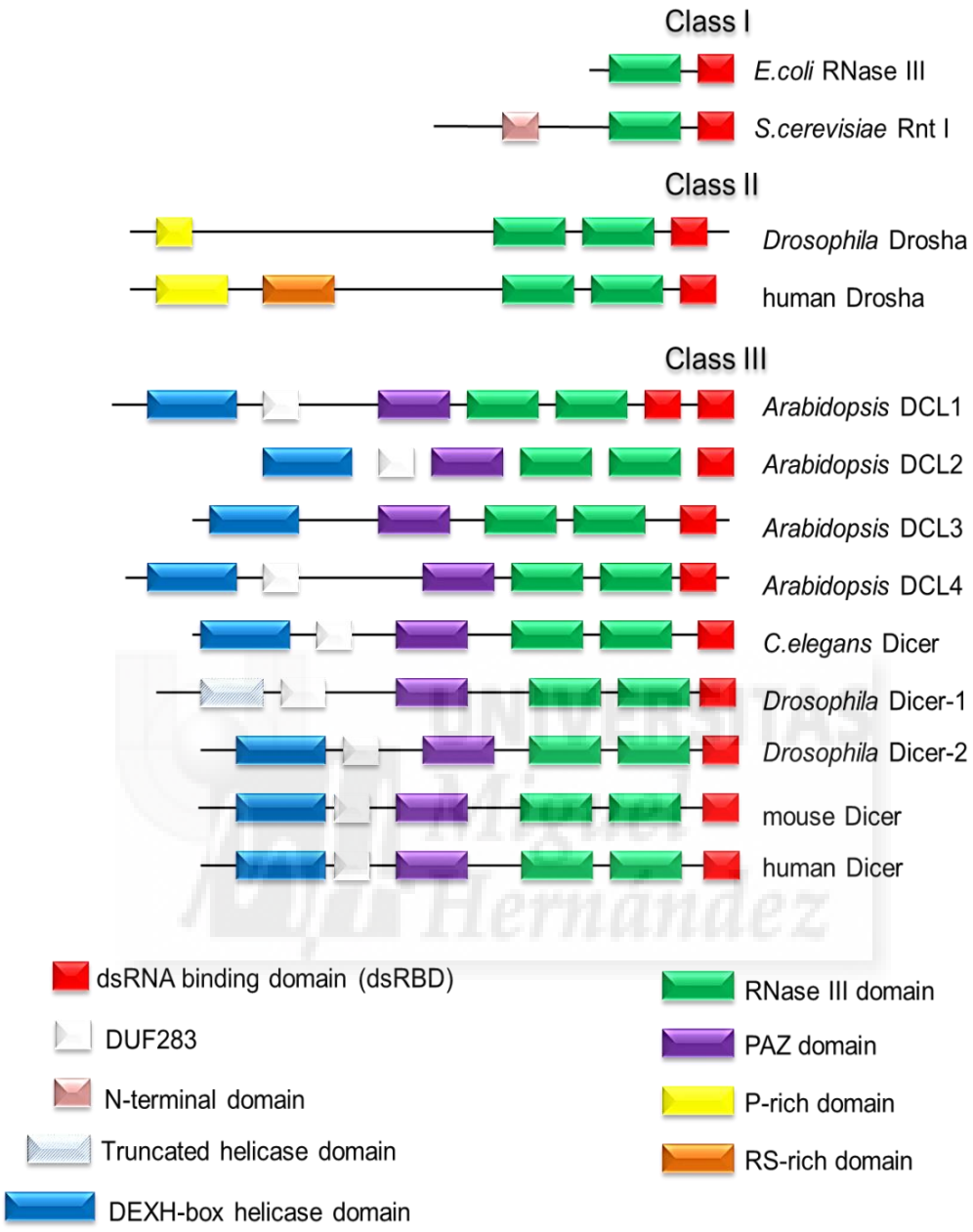

Figure 4. Structural characteristics of RNase III family members. The family is subdivided into three classes based on domain organization. Class I enzymes are found only in bacteria and simple eukaryotes and represented the simplest members. All the RNases III contain a double-strand RNA binding domain (dsRBD) and at least one RNase catalytic domain. Evolution of these enzymes in higher eukaryotes led to the accumulation of additional domains. More details in the text. Adapted from **Johanson et al. 2013**.

1.3.2 Class II RNase III: Drosha

Members of the second class of RNase III proteins, comprised Drosha and homologs, whose function in miRNA biogenesis was previously discussed, is a ~160 kDa protein containing two RNase domains, a dsRBD and a long N-terminal segment (Filippov et al. 2000, Fortin et al. 2002). The N-termini of human and mouse Drosha contain two domains, probably involved in protein-protein interactions, named proline-rich (P-rich) domain and a serine-arginine-rich (RS-rich) domain (Wu et al. 2000, Fortin et al. 2002). Drosha homologs have been identified in flies, worms, humans and mice, but not yet in plants or *Schizosaccharomyces pombe* (Filippov et al. 2000). In addition to the miRNA-related function, Drosha is also able to recognize and cleave mRNAs with characteristic stem-loop structure (Johanson et al. 2013). Human Drosha functions also in processing of highly structured ribosomal RNA precursors, like *E.Coli* RNase III, whereas the conservation of this Drosha ability remains contentious in other species (Wu et al. 2000, Carmell and Hannon 2004, Johanson et al. 2013).

1.3.3 Class III RNase III: Dicer

The third class of RNase III enzymes, comprised of Dicer-like proteins, are ~200 kDa multidomain proteins (**Figure 5a**).

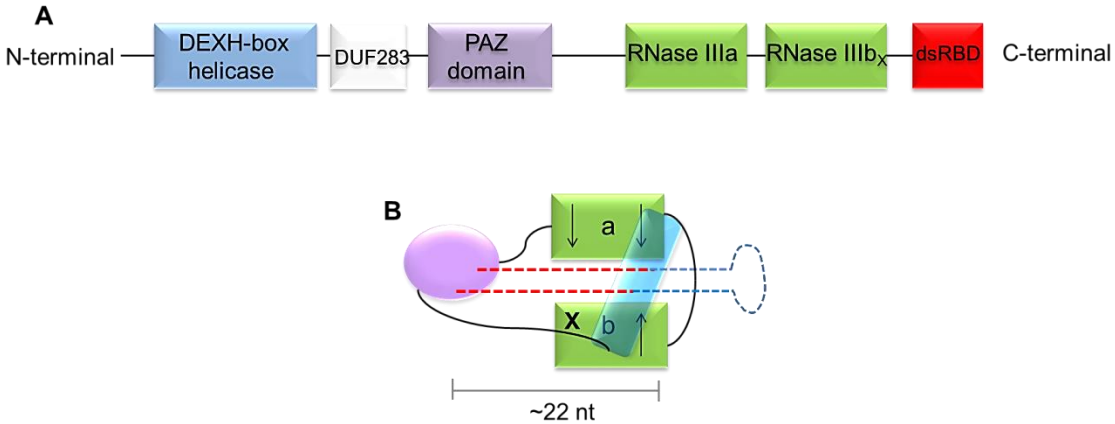

Figure 5. Dicer domains and monomeric model for cleavage. A. Dicer protein domains are represented. **B.** Monomeric model for Dicer cleavage of a pre-miRNA. Arrows indicate catalytic active sites and X indicates a non-functional catalytic site. For clarity dsRBD and DUF283 are omitted. Mature miRNA sequence is indicated in red. More details in the text

As already mentioned, Dicer is an important component of the miRNA biogenesis machinery playing a critical role in miRNA maturation. The name of Dicer was chosen by Emily Bernstein in 2001, a graduate student in Greg Hannon's lab at Cold Spring Harbor Laboratory, who discovered the enzyme responsible for generating small RNA fragments of ~22 nucleotide from double-stranded RNAs in *Drosophila* S2 cells (Bernstein et al. 2001). This enzyme, belonging to the ribonuclease III family and until then referred to as protein CG6493, is responsible for the processing of the vast majority of pre-miRNA in the cytosol leading to the production of mature double-stranded miRNAs. RNase III enzymes were first described in 1968 by Zinder and colleagues (Robertson et al. 1968), exhibit specificity for double-stranded RNAs (dsRNAs) and are found in virtually all eubacteria and eukaryotes, but not archeabacteria (Lamontagne et al. 2001, Liu et al. 2009). Humans and mice have a unique gene codifying for Dicer-1 located respectively on the chromosome 14 and composed of 34 exons and on the chromosome 12 and composed of 34 exons. On the other hand, *Drosophila melanogaster* genome contains two genes codifying for Dicer-1 and Dicer-2 sited in different chromosomes. These two Dicer proteins show different and specialized functions. Indeed, only Dicer-1 is involved in miRNA biogenesis, while Dicer-2 is required for siRNA-directed mRNA cleavage (Lee et al. 2004).

Typically, Dicer enzymes share a core structure consisting in an N-terminal DEXH-box helicase domain, a DUF283 domain with unknown function, a PAZ domain, two in tandem RNase III catalytic domains and one C-terminal dsRNA binding domain (dsRBD) (two dsRBD are present in *Arabidopsis* DCL1). The PAZ domain is a highly conserved module of 130 amino acids, found in Dicer and members of the Argonaute family and is in fact named after three founding Argonaute proteins, Piwi, Argonaute and Zwille. The PAZ domain functions as a molecular sensor, positioned ~65 Å from the RNase III catalytic sites, which specifically recognizes the 2 nucleotides 3′-overhangs at the base of the stem of precursor miRNAs or of a processed dsRNA

terminus (Macrae et al. 2006), a mechanism called as “3’-counting”. As previously mentioned, Dicer protein functions as a monomer and reorganizes its structure to form an intramolecular dimer between its two RNase III catalytic domains to form active sites across the interface of the dimer (**Figure 5b**). It is important consider that only one RNase III domain contains two canonical active site whereas the other one is characterized by the presence of a unique catalytic active site (Blaszczyk et al. 2001). This leads to the formation of a unique cleavage site for Dicer monomer. Importantly, Dicer-mediated cleavage provides precisely-sized RNA products of ~21-24 nucleotides. The precise product length derives from the physical spacing and functional cooperation of the PAZ and RNase III domains. Indeed, Dicer seems to be able to measure from the mono-phosphorylated 5′ end of the precursor to position the RNase III domains for intramolecular dimerization and cleavage approximately 21–24 nucleotides away (Park et al. 2011) in a mechanism known as “5’-counting”. A recent electron microscopy (EM) reconstitution of human Dicer revealed that the helicase domain is in the vicinity of the nuclease domains and may contribute to recognition of the loop region in miRNA precursors (Lau et al. 2012). So, in addition to the “3’-counting” (Macrae et al. 2006) and “5’-counting” (Park et al. 2011) mechanisms for Dicer target site recognition, a ‘loop-counting’ mechanism was proposed for target site selection, in which the helicase domain establishes an additional interaction with the RNA loop (either as a terminal or internal loop) conferring an additional level of cleavage precision not afforded by the PAZ domain alone (Gu et al. 2012). The final product of this Dicer-mediated cleavage of a hairpin-like pre-miRNA is a mature double-stranded miRNA without the stem-loop structure characteristic of its precursor. This step from precursor to mature could be regulated in a many different ways (Finnegan and Pasquinelli 2013). Specific precursor miRNAs can be detained in the nucleus to prevent maturation in certain cell types (Lee et al. 2008). Precursor maturation can also be regulated globally by affecting Dicer levels and activity. Human Dicer mRNA is itself a target of miRNA repression via three conserved let-7 binding

sites within its coding sequence (Forman et al. 2008). Additionally, Dicer protein negatively regulates its own catalytic activity through its helicase domain (Ma et al. 2008). This auto-inhibitory effect may be modulated by binding of Dicer cofactors to the helicase domain. Thus, Dicer activity can be altered through many protein interactions. One Dicer interactor that increases cleavage efficiency is the HIV-1 TAR RNA-binding protein (TRBP). While Dicer is capable of cleaving precursor miRNAs into their mature forms without any cofactors, TRBP enhances this reaction in mammalian cells (Ma et al. 2008, Chakravarthy et al. 2010, Koscianska et al. 2011). TRBP is an approximately 45-kDa RNA binding protein containing three dsRBDs. Two of the dsRBDs can homodimerize or bind to the interferon (IFN)-induced protein kinase R (PKR) and the protein activator of PKR kinase (PACT) by the reciprocal interaction of dsRBDs. The third dsRBD interacts with the N-terminal helicase domain of Dicer in a RNA-independent manner (Haase et al. 2005, Lee et al. 2006, Laraki et al. 2008). The exact role TRBP plays in precursor cleavage remains under debate. Some studies have found that the TRBP and Dicer interaction stabilizes Dicer protein (Chendrimada et al. 2005, Koscianska et al. 2011) and the Dicer-substrate complex (Chakravarthy et al. 2010). Alternatively, TRBP may assist in recruiting substrates to Dicer (Parker et al. 2006, Parker et al. 2008, Chakravarthy et al. 2010). Based on the biochemical results (Castanotto et al. 2007) and data from electron microscopy imaging (Wang et al. 2009), TRBP could also acts like a sensor for proper strand loading to RISC, which can proofread incorrect strand loading. The dsRNA binding proteins, protein activator of PKR (PACT) in mammals and Loquacious (Loqs) in Drosophila, also associate with Dicer and facilitate processing of miRNA precursors (Forstemann et al. 2005, Jiang et al. 2005, Saito et al. 2005, Lee et al. 2006). PACT and TRBP associate directly with each other as well as with Dicer in the cytoplasm (Lee et al. 2006, Kok et al. 2007). Depletion of PACT or TRBP in mammalian cells results in reduced mature miRNA levels, indicating that the two factors, although similar, are not entirely redundant (Haase et al. 2005, Lee et al. 2006, Chakravarthy et al. 2010, Koscianska et al. 2011).

It has been suggested that these Dicer cofactors help synchronize the dual cleavage by each of the RNase III domains (Koscianska et al. 2011). Apart from the critical role in miRNA biogenesis pathway, Dicer also has important roles in genome regulation and surveillance. The peculiar position of the helicase domain respect to the RNase III domain and its ability to engage the dsRNas also confers processivity to Dicer enzymatic action, since engagement of dsRNA allows successive rounds of cleavage prior to release of substrate (Lau et al. 2009). This ability is crucial for allowing the miRNA-independent Dicer roles. Indeed, in addition to its central role in miRNAs biogenesis, Dicer is able to produce endogenous small interfering RNAs (endo-siRNAs) from numerous sources of dsRNA, including small nuclear RNAs (Langenberger et al. 2010) and small nucleolar RNAs (Ender et al. 2008, Taft et al. 2009, Brameier et al. 2011). Dicer can also process viral sources of dsRNAs to produce viral siRNAs involved in various anti-viral silencing responses (Ding 2010). In addition to endo-siRNA-mediated silencing, cleavage by Dicer represents a second mechanism for detoxifying repeat-element-derived transcripts, such as those containing short interspersed elements (SINEs). Dicer appears to detoxify SINE RNAs by degradation into small 25-50 nucleotides-long RNAs, products larger than the typical ~21-24 nucleotides-long miRNAs and siRNAs (Kaneko et al. 2011). Finally, Dicer, together with Drosha and in a miRNA-independent manner, plays important protective functions in the response of cells to DNA damage. In fact, these two RNases III are responsible for the production of both DNA damage-induced miRNAs and DNA-damage-induced small RNAs (DDRNAs) or double-stranded break-induced RNAs (diRNAs) (Lee et al. 2009, Francia et al. 2012, Chowdhury et al. 2013). This last type of small RNAs, first observed in the fungus *Neurospora crassa* but also detected in more complex organisms, including plants, flies and vertebrates (Lee et al. 2009), appear to derive from sequences near the dsDNA break (Wei et al. 2012) but their precise mechanism of action remains unclear. One possibility is that DDRNAs/diRNAs function guiding chromatin-modifying complexes to sites of DNA damage inducing gene

silencing (Lukas et al. 2011). In alternative, because of sequence homology, it is also possible that DDRNAs/diRNAs guide DNA repair machinery to the site of damage in a manner similar to miRNAs/siRNAs guiding the RISC to target mRNAs (Johanson et al. 2013).

In spite to its important functions, only one pathological condition is directly associated with mutation in human *Dicer-1* gene. *DICER1* syndrome, also named *DICER1*-related disorders and *DICER1*-Pleuropulmonary Blastoma Familial Tumor Predisposition Syndrome, is a a familial tumor susceptibility syndrome inherited in an autosomal dominant manner that confers increased risk most commonly for pleuropulmonary blastoma (PPB), ovarian sex cord-stromal tumors (Sertoli-Leydig cell tumor), juvenile granulosa cell tumor (JGCT) or gynandroblastoma, cystic nephroma (CN) and thyroid gland neoplasia (multinodular goiter [MNG], adenomas, or differentiated thyroid cancer. Somatic missense pathogenic variants appear to preferentially affect amino acids in the RNase IIIb domain (1705, 1709, 1809, 1810 or 1813) and are characterized as “missense hotspots”. These somatic pathogenic variants lead to defective production of mature miRNAs from the 5’ (5p) end of the miRNA hairpin but preserve the cleavage of the 3’ (3p) end of the hairpin (Gurtan et al. 2012, Anglesio et al. 2013). No direct associations have been observed between *Dicer-1* gene mutations and neurological diseases.

1.4 MiRNA function

Although different biogenesis pathways, all miRNAs share some features, such as the pre-miRNA hairpin and the seed sequence for the target mRNA recognition. The seed sequence is defined like the region at the 5’-end of a miRNA spanning the nucleotides 2-8 that is responsible to the recognition of target mRNAs by pairing with a complementary region in its 3’UTR named as MiRNA Recognition Element (MRE). The

limited sequence complementarity between the miRNA and its target allows a single miRNA to regulate many mRNAs targets. Thus, once loaded into the Ago protein of the silencing complex, miRNAs pair with target mRNAs to direct posttranscriptional repression, generally by blocking translation. In metazoan, the base pairing between miRNA and its target is usually imperfect, showing a bulge or mismatch in the central region of the miR:mRNA duplex that preclude an Ago-mediated endonucleolytic cleavage of the mRNA. Translational repression could occur by interfering with the initiation step, slowing the elongation or the ribosome "drop-off" or inducing the proteolytic cleavage of the nascent polypeptide (Filipowicz et al. 2008). In contrast with this initial view, more recent works demonstrated that the miRNA-mediated repression is frequently associated with mRNA destabilization (Bagga et al. 2005, Behm-Ansmant et al. 2006, Giraldez et al. 2006, Wu et al. 2006). In eukaryotes, miRNA-mediated mRNA degradation is always initiated by a gradual shortening of the mRNA poly(A) tail operated by the CCR4-NOT deadenylating complex but can then follow two different mechanisms. Indeed, the mRNA sequence can be degraded by progressive 3'→5' decay, catalysed by exosome, or by removal of the cap followed by 5'→3' degradation, catalysed by the exonuclease XRN1. The degradation, or at least its final steps, is thought to occur in P-bodies, cellular structures that are enriched in mRNA-catablizing enzymes and translational repressors (Filipowicz et al. 2008). Nevertheless, although many of the mRNAs that are targeted by miRNAs undergo substantial destabilization, there are also numerous examples of repression at the translational level, with no or only a minimal effect on mRNA decay. It is not known what determines whether an mRNA follows the degradation or translational-repression pathway. Accessory proteins bound to the 3′ UTR might be involved, or structural subtleties of imperfect miRNA–mRNA duplexes, particularly of their central regions, could be important (Filipowicz et al. 2008). Importantly, these two miRNA-mediated repression mechanisms, based on mRNA decay or protein synthesis blocking, are not mutually exclusive, but likely operate in a combinatorial manner (Filipowicz et al. 2008, Bartel 2009). To complicate

this issue, non-canonical MREs have been identified, such as those in 5'UTR, coding regions or seedless sites, and multiple MREs may be present within a single 3'-UTR suggesting an added level of complexity where target regulation may involve cooperative and/or combinatorial regulation of multiple miRNAs with an individual transcript (Bartel 2009, Vo et al. 2010). Depending on whether the dampening of protein output is beneficial, inconsequential, or harmful, the MRE sites are either selectively conserved, neutral, or selectively avoided during evolution, with the mRNAs classified as conserved targets, neutral targets and antitargets, respectively, of the miRNA.

1.4.1 miRNAs:target interactions

Different types of binary miR:mRNA interactions have been observed, each one characterized by different effects on protein output (**Figure 6**). The classical interaction model is the switch interaction. This type of interaction has probably the stronger effect on protein output, turning off the target to inconsequential levels, thus unable to result in significant effect or function. The switch interaction between miRNAs and their targets is particularly important and useful, for example, to determine and/or maintain cell and tissue identity repressing the expression of genes that should not be expressed in a particular cell type. The miRNA could be just present before its target is first expressed or induced in response to its increased and close to functionally consequential levels (**Figure 6A**). An example is the repression of *senseless* by miR-9a in epithelial cells of *Drosophila* preventing the sporadic production of extra neuronal precursor cells (Li et al. 2006). At their extreme, such switch interactions can be regarded as failsafe interactions, differing because protein output falls below functional levels even in the absence of the miRNA. For failsafe interactions, miRNA repression adds an additional, functionally redundant layer of repression, helping ensure that aberrant transcripts do not give rise to a consequential amount of protein (Bartel 2009).

The miR-1-mediated repression of non-muscle Tropomyosin isoforms and non-muscle V-ATPase subunits in the developing muscle is an example of this last type of interaction (Stark et al. 2005).

A second type of interaction is known as “tuning interaction”, which are those in which the miRNA acts as a rheostat rather than a binary off-switch to dampen protein output to a more optimal level but one that is still functional in the cell (**Figure 6B**), thereby enabling more customized expression in different cell types as well as more uniform expression within each cell type (Bartel 2009). An example of tuning interaction is the *Drosophila* miR-8 regulation of *atrophin*, which reduces protein output to a level that prevents neurodegeneration but not as low as to compromise viability (Karres et al. 2007).

Finally, neutral interactions dampen protein output but this repression is tolerated or offset by feedback mechanisms such that the regulatory sites are under no selective pressure to be retained or lost during the course of evolution (**Figure 6C**) (Bartel 2009). Neutral interactions comprise cases in which the interaction has no biological function and is due to accidentally pair between mRNAs and co-expressed miRNAs. Considering the degree of target repression, with the exception of neutral interaction, the switch interactions are thought to confer strongest repression, whereas the tuning interactions induce more modest effects (Bartel 2009). In fact, although many miRNAs and their target binding sites are deeply conserved, suggesting an important role, a typical binary tuning miRNA/mRNA interaction produce only subtle reduction (<2-fold) in protein levels (Baek et al. 2008). Nonetheless, these modest changes could be amplified if the miRNA-mediated regulation cooperates with transcriptional regulation to achieve classical switch function. This effect occurs when a miRNA targets a mRNA whose transcription has just shut off. In this case, depending on the threshold level for protein function, mRNA decay rate and protein decay rate, a modest miRNA-mediated repression can lead to significantly reduced protein at later time points, with a much more rapid transition to the off state (**Figure 6D**) (Bartel 2009).

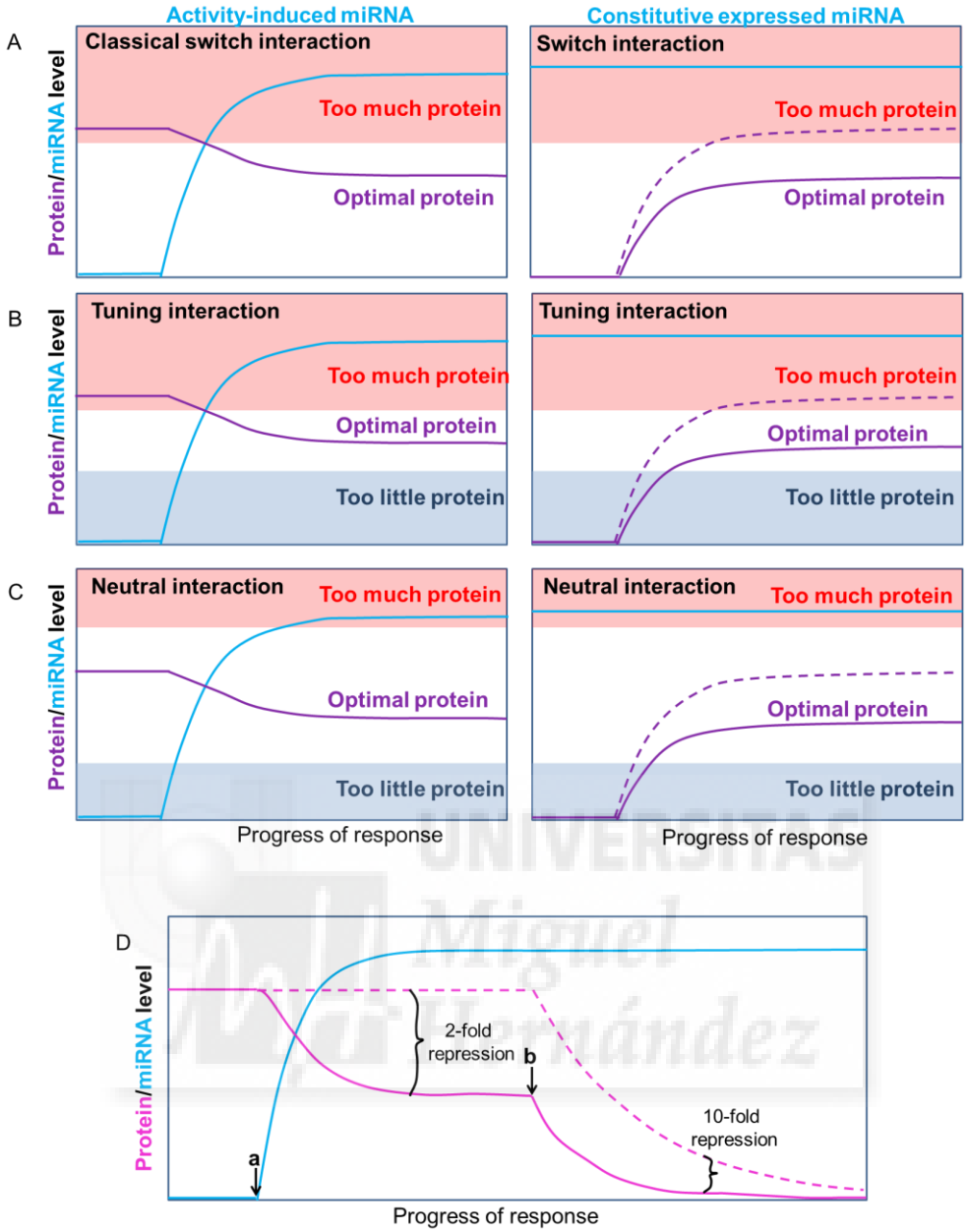

Figure 6. Effects of miRNA regulation on protein level. A-C. Different types of miRNA interactions. **A.** Switch interaction. **B.** Tuning interaction. **C.** Neutral interaction. In the left panels, miRNA expression (in blue) is induced in response to environmental or developmental cue and the level of the protein (in purple) produced from its mRNA target is reduced. In right panels, protein levels are reduced by pre-existing miRNA. Protein accumulation is represented by dashed line. The magnitude of repression shown in these panels is an example given that really could be variable due to target properties, such as the threshold for functional protein. More information about miRNA interactions in the text. **D.** Modest destabilization induces greater repression after mRNA transcription stopping. A miRNA is induced (a) (in blue) and mediates modest mRNA destabilization (in pink). After the stop of the transcription of the target (b), the same modest destabilization is able to quickly induce a strong repression of protein output (≥10-fold). If the miRNA also mediates translational repression, the transition to the off state is accelerated. Figure adapted from **Bartel 2009**.

Many conserved mammalian interactions involve targets that are not strongly repressed by the miRNA and are presumed to be tuning interactions (Bartel 2009).

To elucidate the role of miRNAs, it could be interesting considering the relative expression of miRNAs and their targets. Global gene expression analyses in different species have shown that miRNAs and their targets often have mutually exclusive expression, likely the result of switch interactions, across tissues, especially in neighbouring tissues derived by common progenitors (Farh et al. 2005, Stark et al. 2005), suggesting that miRNAs can reinforce developmental gene expression program by repressing leaky transcripts ensuring tissue identity and supporting cell-lineage decision using a variety of circuit motifs to regulate the gene expression networks, such as feedback and feed-forward loops (Tsang et al. 2007). In contrast to development, in adult neurons, neuronal-enriched miRNAs tend to be co-expressed with their target genes, suggesting the involvement of these miRNAs in neuronal homeostasis (Tsang et al. 2007) via tuning interactions.

Considering the aforementioned binary interactions between miRNAs and their targets and in spite of the large numbers of target genes predicted to be affected by miRNA loss of function, gene knockout experiments for individual miRNAs have produced many disappointing results. For instance, in *C. Elegans* most individual miRNA mutants show no gross phenotype (Miska et al. 2007) and the same occurs in several mouse knockouts generated to date, including miRNA-21, miRNA-210, miRNA-214, miRNA-206 and miRNA-143 (Ebert and Sharp 2012). One of the more interesting views to interpret why such perturbations are so well tolerated, even for miRNA targets that are themselves gene-regulatory proteins, is the phenomenon of regulatory network buffering. Many regulatory interactions, including many miRNA:target interactions, presumably fall within complex regulatory networks with bifurcating pathways and feedback control that enable accurate response despite a defective node in the network. Another partial explanation for these results is the functional redundancy of some miRNAs that share the same seed sequence forming a “seed family”. An

example is represented by let-7 family members miRNA-48, miRNA-84 and miRNA-241 that operate redundantly in the control the L2-L3 larval transition in *C. elegance* (Abbott et al. 2005). In addition, miRNAs of different "seed family" could also work together co-targeting the same gene or set of gene and providing overlapping function (Ebert and Sharp 2012). An additional explanation for the lack of effects in knockout animals for a single miRNA could be that the mutant phenotype is visible only after acute miRNA deletion if, during development, miRNA loss can be compensated. This is the case of Drosophila mutants hypomorphic for important miRNA pathway components, such as *Drosha*, *Pasha* and *Dicer-1*, which loss is generally lethal in animals, that only result in a reduced synaptic transmission in adult mutant photoreceptor neurons without any defects in neuronal development or maintenance (Smibert et al. 2011). Another possibility to explain the loss of evident phenotypes is that the effect deriving from the lack of a single microRNA could be just observed upon the application of certain internal or external stresses. For example, knockout mice for the miRNA-208 specifically in heart-muscle showed no gross phenotypical effects in normal conditions but an important defect in the induction of cardiac remodelling after stress (van Rooij et al. 2007). Taken together all the observations above mentioned, it seems likely that miRNAs could help to confer robustness to biological processes acting like a buffering system that prevents fluctuations in gene expression, thereby contributing to maintain regulatory networks (Ebert and Sharp 2012). In any case, more studies are still required to elucidate the exact role of miRNAs.

1.5 Molecular tools to investigate miRNA function

The best way to study the functional relevance of a miRNA is by examining phenotypic changes in culture or within an organism in response to regulation of a miRNA. Recently, several strategies for gain- and loss-of-function studies for specific miRNAs both *in vitro* and *in vivo* have been developed. To overexpress an individual miRNA, it's

possible to transfect in many types of cultured cells miRNA mimics, usually small chemically modified double-stranded RNAs that mimic mature endogenous miRNAs, to infect *in vitro* or in vivo with viral vectors carrying on pre-miRNA sequences or use transgenesis to generate miRNA overexpression in specific cell type using specific promoter. Although this approach has proven useful in defining the function of several miRNAs, forced overexpression of a miRNA can potentially result in the regulation of physiologically irrelevant targets if the transgene reaches supra-physiological levels of expression. A more elegant way to study the functional relevance of a miRNA is by downregulation or genetic deletion. Several examples of miRNA knockout animals have now been published and have revealed very specific functions for the deleted miRNAs, especially under diseased conditions (Zhao et al. 2007, Hebert et al. 2008, Remenyi et al. 2013, Dahan et al. 2014, Papadopoulou et al. 2014, Hernandez-Rapp et al. 2015, Kramer et al. 2015). Although these models often provide valuable insights, it`s important to consider that genetic deletion of a single miRNA might not result in a phenotypic effect because of redundancy with related miRNAs and that, in some cases, the genetic deletions might be compensated for over the course of a lifetime.

Two important tools to induce miRNA loss of function are represented by miRNA sponges and antimiRs. MiRNA sponges are defined like competitive inhibitors of miRNAs in mammalian cells (Ebert et al. 2007, Ebert and Sharp 2010). These constructs, that can be viral delivered, contain multiple target sites complementary to a miRNA of interest that specifically “soak up” miRNA levels (Ebert et al. 2007, Ebert and Sharp 2010). Importantly, the miRNA binding sites have a bulge at the position normally cleaved by Ago2 to augment the stability of the interaction between miRNA and its sponge (Ebert et al. 2007, Ebert and Sharp 2010). The other approach to repress miRNA levels and function is the use antimiRs, chemically modified antisense oligonucleotides that pair with and block mature miRNAs through extensive sequence complementarity. There are several key requirements for an antimiR chemistry to

achieve effective downregulation of a targeted miRNA *in vivo*. The chemistry needs to be cell-permeable, cannot be rapidly excreted, needs to be stable in vivo, and should bind to the miRNA of interest with high specificity and affinity (Manoharan 1999, Prakash and Bhat 2007, Stenvang and Kauppinen 2008). Several modifications have been used *in vivo* to date. These chemical modifications include 2′-O-methyl group–modified oligonucleotides and locked nucleic acid (LNA)-modified oligonucleotides, in which the 2′-O-oxygen is bridged to the 4′ position via a methylene linker to form a rigid bicycle, locked into a C3′-endo (RNA) sugar conformation (Weiler et al. 2006). Another chemical modification applied to enhance oligonucleotide stability is the balance between phosphodiester and phosphorothioate linkages between the nucleotides, with phosphorothioate providing more stability to the oligonucleotide and making it more resistant to nucleases. The 2′-O-methyl group modification is used most often to improve nuclease resistance and improve binding affinity to RNA compared with unmodified sequences. In 2005, Krutzfeldt et al reported on the first mammalian in vivo study using these so-called “antagomirs”, 2’ O-methyl, phosphorothioate, cholesterol-modified antisense nucleotides, to inhibit miR-122, a liver-specific miRNA (Krutzfeldt et al. 2005). These chemically modified oligonucleotides are complementary to the mature miRNA sequence and are conjugated to cholesterol to facilitate cellular uptake. Systemic delivery of an antagomir via intravenous injection is sufficient to efficiently reduce the level of the miRNA of interest in multiple tissues for an extended period of time and resulted in upregulation of genes involved in cholesterol biosynthesis. Although the required doses are quite high, a single intravenous bolus injection of an antagomir is sufficient to inhibit the function of its target miRNA for weeks. These lines of evidence validate the efficacy of antagomirs *in vivo* and probably founded the basis for the antagomir being the most commonly used antisense oligonucleotide to silence miRNAs in research studies thus far. Recently, the therapeutic applicability of LNA-antimiR technology has been reported in rodents and nonhuman primates. A locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide

carrying the ribose modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo conformation that enhances base stacking and backbone pre-organization. The LNA modification leads to a thermodynamically strong duplex formation with complementary RNA, and systemic delivery of unconjugated LNA-antimiR potently antagonized the liver-expressed miR-122 in mice and nonhuman primates (Elmen et al. 2008a, Elmen et al. 2008b).

2. MiRNAs in the brain

The nervous system is an intricate set of circuits formed by different cellular types that allows a subject to receive and respond to internal and external stimuli conferring the ability to adapt to the environment and store information. In order to achieve this conserved but dynamic structure, the nervous system undergoes extensive changes in patterning, remodelling and cell fate specification during development, when originally stem cell populations generate thousands of different neuronal and glia cell types in a temporally and quantitatively perfectly orchestrated manner. The importance of miRNAs in the regulation of brain development has been extensively demonstrated (Reinhart et al. 2000, Bernstein et al. 2003, Krichevsky et al. 2003, Miska et al. 2004, Giraldez et al. 2005, Harfe et al. 2005, Vo et al. 2005, Boutz et al. 2007, Makeyev et al. 2007, Miska et al. 2007, Visvanathan et al. 2007, Choi et al. 2008, Davis et al. 2008, Kawase-Koga et al. 2009, Li and Piatigorsky 2009, Gao 2010, Georgi and Reh 2010, Iida et al. 2011, Li et al. 2011, Li et al. 2012, Yao et al. 2012, Harraz et al. 2014, Petri et al. 2014).

After their generation, young neurons have to connect with pre-determined target neurons through the establishment of functional specialized junctions, known as synapses, either in their immediate environment or at distance. However, the function of the adult brain depends not only on static circuitries but also on plastic changes at

the synaptic level shaped by sensory experience allowing complex processes, such as learning and memory. In the mammalian adult brain, neurons are interconnected by trillions of specialized synapses and thus it appears evident that all these plastic processes, that need flexibility and stability at the same time, have to be under the control of complex molecular networks that ensure a fine-tuning regulation of gene expression programmes that would control, in turn, the long-lasting alteration of synapses in response to environmental stimuli. Among these mechanisms and their temporal regulation, the local control of mRNA translation in neuronal dendrites can account for the tight spatial regulation of plasticity at the level of individual dendrites or spines (Sutton and Schuman 2006). Many evidences suggest that miRNA system is involved in the control of local translation in dendrites.

Given the cellular and transcriptional complexity of the nervous system, it is not surprisingly that miRNAs are highly abundant in the brain (Krichevsky et al. 2003, Miska et al. 2004, Sempere et al. 2004) affecting a large number of neuronal genes (Lagos-Quintana et al. 2002, Krichevsky et al. 2003, Kim et al. 2004). The number of miRNAs expressed in the nervous system seems to be larger if compared with other organs or tissues, probably reflecting a complex cellular composition that includes numerous types and subtypes of cells. Although initial miRNAs expression analysis were limited to broad regions of brain demonstrating that a similar set of miRNAs was expressed but with significant different relative expression levels (Lagos-Quintana et al. 2002, Kim et al. 2004, Sempere et al. 2004, Baskerville and Bartel 2005, Berezikov et al. 2006), recent studies based on new profiling technologies show that spatial miRNA expression pattern is highly complex reflecting the wide variety of neuronal types (He et al. 2012). In addition, miRNAs are expressed in neuronal and non-neuronal cells in the brain, such as oligodendrocytes and glia where they are also likely to perform important functions (Shin et al. 2009, Bremer et al. 2010, Budde et al. 2010, Dugas et al. 2010,

Pereira et al. 2010, Verrier et al. 2010, Dugas and Notterpek 2011, Gokey et al. 2012, Li and Yao 2012, Lin et al. 2013).

Key features of neuronal miRNAs include their ability to regulate mRNA translation locally in the synapto-dendritic compartment, the modulation of their expression and function by neuronal activity and the capacity of a single or few miRNAs to control simultaneously the expression of several synaptic transcripts (Schratt 2009a).

2.1 Subcellular compartmentalization

Even within a single neuron, complex functional architecture offers many compartments that could be regulated by different sets of miRNAs. An early comparison between miRNAs in the cell body and in neurites of rodent hippocampal neurons showed a graded distribution across a set of 99 candidates identified by laser capture and multiplex RT-PCR, the extremes of which defined miRNAs that are selectively enriched in dendrites versus soma (Kye et al. 2007, McNeill and Van Vactor 2012). In spite of that, the synaptic compartment seems to contain a large fraction of the total neuronal miRNAs (Lugli et al. 2008). Recent analysis of the distribution of miRNAs in five rat brain region (cortex, hippocampus, cerebellum, brainstem and olfactory bulb) demonstrates a selective enrichment of miRNAs in synaptic compartment (Pichardo-Casas et al. 2012) containing the ~79-97% of the total miRNA pool expressed. The dendritic and synaptic localization of miRNAs in the synapto-dendritic compartment might contribute to the regulation of neuronal function through their ability to control local translation. In particular long-term synaptic plasticity is known to require local protein synthesis (Kandel 2001, Sutton and Schuman 2006). Supporting this hypothesis, it has been shown that miRNAs-containing dendritic granules respond to neuronal activation, suggesting that miRNAs might participate in

the local and dynamic control of mRNA translation in dendrites (Cougot et al. 2008, Mollet et al. 2008).

An important question that arises from the demonstration of the presence of active dendritic miRNAs is how they translocate to their final localization in dendrites. Based on research of dendritic transport of mRNAs, two alternative but not exclusive models have been postulated. The first possibility is that the miRNA is co-transported bound with its target mRNA that contains dendritic localization signals (Steward and Schuman 2001, Zhong et al. 2006). Alternatively, dendritic miRNAs might be processed to its mature form in the dendrite, requiring that the pre-miRNA, Dicer and the RISC complex components are transported to the dendrite. Supporting this view, Dicer and Argonaute proteins, component of RISC, are detected and enriched in dendrites (Lugli et al. 2005, Barbato et al. 2007). According to this last model, miRNAs have a limited "narrowed" mRNA target field because only the mRNAs present in the dendrite can be targeted (Kosik 2006). Although the strong interest in studying miRNA system and its neuronal function, to date none of models have been experimentally demonstrated.

2.2 Activity-dependent regulation

Neurons are able to adapt their response to environmental changes due to the possibility to regulate gene expression programmes by neuronal activity. Like for other important neuronal regulators of activity-dependent gene expression, such as transcription factors and co-factors, the function of several miRNAs can be regulated by neuronal activity (**Figure 7**). This regulation, crucial to couple neuronal miRNAs activity with synaptic function, could occur to multiple levels (Schratt 2009b). A first possibility is that activity controls the transcription of miRNAs (**Figure 7a**). Several promotors of neuronal miRNAs are occupied by classical activity regulated transcription

factors, such as CREB and MEF2, able to combine Ca^{2+}-dependent signalling cascade with transcription (Vo et al. 2005, Fiore et al. 2009). In a genome-wide screening for CREB binding sites, several consensus binding sites were detected near to the miR132/miR212 locus and the miRNA-132 was first identified like a neuronal enriched miRNA regulated by neuronal activity (Vo et al. 2005). In fact, the production of the transcript for pri-miRNA-132 is induced in response to several and different stimuli in primary cultured neurons, such as BDNF (Vo et al. 2005, Remenyi et al. 2010), KCl and bicuculline (Wayman et al. 2008). These observations obtained *in vitro* have been also confirmed by *in vivo* experiments (Nudelman et al. 2010, Eacker et al. 2011, Mellios et al. 2011, Tognini et al. 2011, Wang et al. 2013).

The regulation by activity of the transport of miRNAs or their precursor to the dendrite could be an alternative mechanism (**Figure 7b**). To the date, no evidence has been presented supporting an activity-regulated miRNA transport. Nevertheless, several studies have demonstrated the existence of activity-dependent transport of RNA granules that, as mentioned above, could contain miRNAs or pre-miRNAs (Antar et al. 2004, Antar et al. 2005, Piazzon et al. 2008, Pascual et al. 2012, Charalambous et al. 2013). Supporting this view, at least two different studies have demonstrated that fragile X mental retardation proteins (FMRPs), normally present in RNA granules and interacting with components of the RISC complex (Caudy et al. 2002, Ishizuka et al. 2002), are associated with several miRNAs (Edbauer et al. 2010, Kenny et al. 2014).

A third possibility is that pre-miRNA processing occurs in dendrites in an activity-dependent manner (**Figure 7c**). Biochemical purification of RISC components from synaptosomes shows detectable levels of pre-miRNAs (Lugli et al. 2008) whereas *in situ* hybridization experiments detected specifically miRNAs in the dendritic compartment (Bicker et al. 2013). In addition, Dicer, the RNase responsible for pre-miRNA processing, is also enriched in synaptosomes and its activity is stimulated by calpain cleavage in an activity-dependent manner (Lugli et al. 2005), suggesting an

enhanced miRNAs production at activated synapses. Finally, miRNA-associated proteins can be also regulated by neuronal activity affecting the ability of miRNAs to regulate target gene expression (**Figure 7d**).

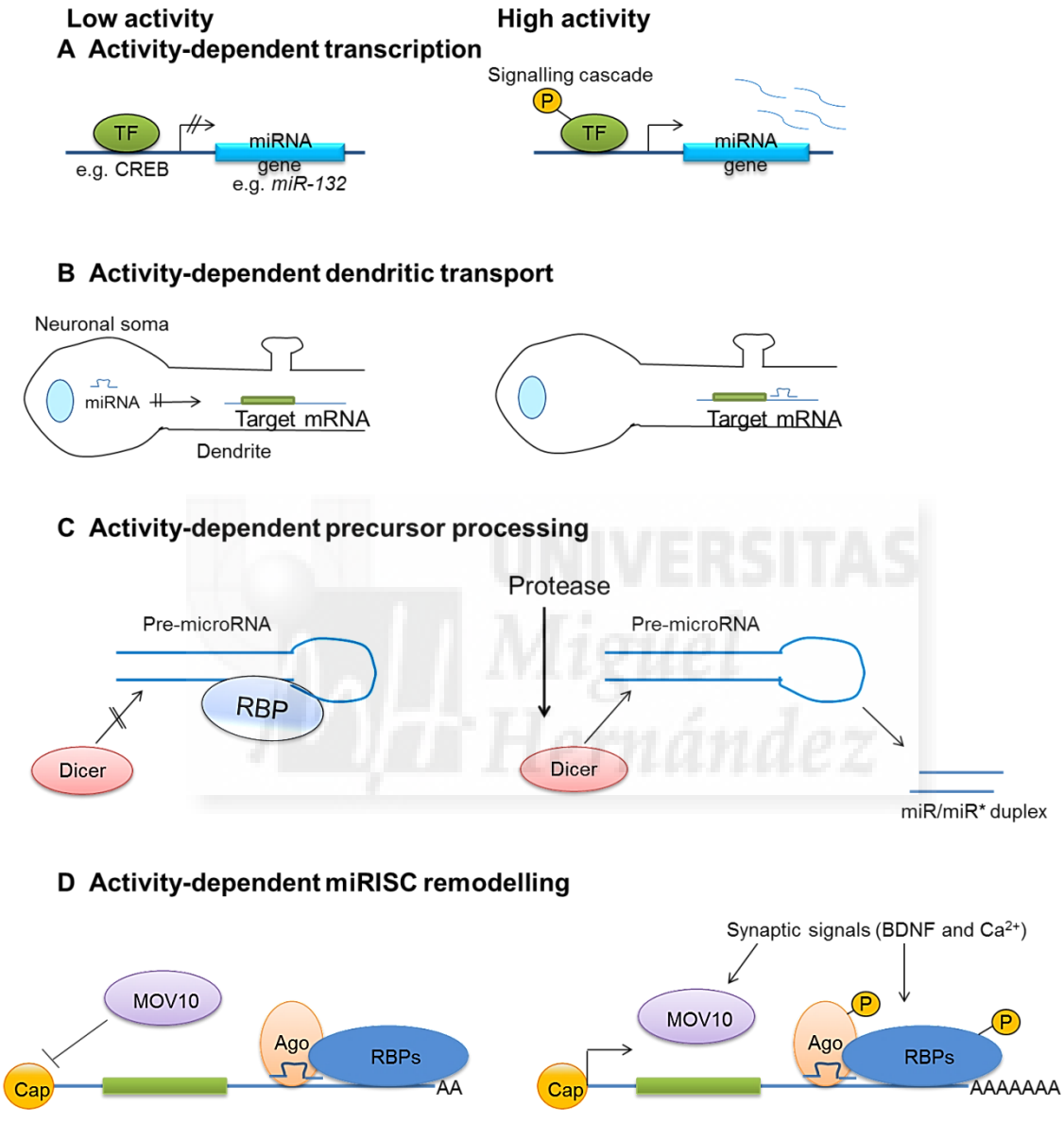

Figure 7. miRNA regulation by neuronal activity. Several neuronal miRNAs are subject to regulation by neuronal activity at multiple levels. Whereas activity-dependent transcription (**A**) and miRNA-induced silencing complex (miRISC) remodelling (**D**) are experimentally supported, the regulation of miRNA dendritic transport (**B**) and processing (**C**) by activity is not demonstrated. For more details, see the text. Adapted from **Schratt 2009**.

For example, synaptic stimulation can inactivate or completely disrupt components of miRNA-induced silencing complex (miRISC) like the *Drosophila* protein Armitage (Armi) and its mammalian homolog MOV10 (Ashraf et al. 2006, Banerjee et al. 2009). In the case of Drosophila, Armi is degraded via the ubiquitin proteasome following activation of the nicotinic acetylcholine receptor. In mammals, MOV10 is also degraded in a proteosomal-dependent manner in response to NMDA receptor activation (Banerjee et al. 2009, Jarome et al. 2011). In neurons, degradation of MOV10/Armi reduces miRNA-mediated repression by an unknown mechanism, allowing for the translation of mRNAs involved in synaptic plasticity (Ashraf et al. 2006, Banerjee et al. 2009). Although biochemical data suggest that Armi/MOV10 is required for the loading of AGO with mature miRNAs (Tomari et al. 2004), recent genome-wide studies have identified numerous promiscuous interactions between polyadenylated mRNAs and MOV10 (Castello et al. 2012, Sievers et al. 2012), suggesting additional potential interactions of MOV10 and miRNA-mediated silencing.

Taken together, all these studies indicate that miRNAs and their associated proteins are important components of the intricate machinery regulating activity-dependent gene expression, which enables the miRNA system to control adaptive processes such as synaptic plasticity. Nevertheless, more research is still required to elucidate the precise mechanisms of the regulation of miRNAs function by neuronal activity.

2.3. MiRNAs and plasticity

Many studies demonstrated that neuronal miRNAs, often located in synapses, are important regulators of activity-dependent synaptic plasticity. Importantly, modification in this type of plasticity results in changes of neuronal excitability, due to both structural changes at the synapses and modification in synaptic strength. Structural changes at

the synapse are commonly associated with alteration in the cytoskeleton function, leading to either the formation of new synapses or the dissolution of pre-existing ones (Bosch and Hayashi 2012, Rochefort and Konnerth 2012). In the vertebrate central nervous system, excitatory synapses are usually formed on small, mushroom-like protrusions on dendrites called dendritic spines (Hayashi and Majewska 2005). It is now accepted that spine morphology is closely related to synaptic structure and functional state. In general, larger, mushroom-shaped spines are associated with larger post-synaptic densities, allowing for more stable and stronger synaptic transmission. This is in contrast to the more dynamic filopodia-type spines, which are associated with weak or absent synaptic transmission (Yoshihara et al. 2009). Several specific miRNAs are reported to be involved in the regulation of synaptic plasticity acting at different levels. For example, excluding the presynaptic miR-485, an activity-induced miRNA that reduces neurotransmitter release by targeting the presynaptic protein SV2A (Cohen et al. 2011), the majority of synaptic miRNAs are detected in the postsynaptic compartment having as primary function the negative regulation and suppression of synaptic connections. One of the first synaptically enriched miRNAs identified, miR-134, was shown to be a negative regulator of synaptic spine volume (Schratt et al. 2006). Its precursor is transported specifically to dendrites via binding to the DEAH-box helicase DHX36 (Bicker et al. 2013). After the pre-miR-134 processing, the miR-134 is released to inhibit spine formation in hippocampal cultured neurons (Schratt et al. 2006) and dendritogenesis in cortical neurons (Christensen et al. 2010) by repressing Limk1 expression, a kinase that regulates spine morphology by regulating ADF/cofilin interactions with the actin cytoskeleton. The reduced spine volume associated with miR-134 overexpression can cause a reduced synaptic strength. Consistent with this hypothesis, mice overexpressing miR-134 show defects in the establishment of long-term potentiation (LTP) in the hippocampus through the inhibition of Sirt1 gene (Gao et al. 2010). Two recent papers have investigated further the role of miR-134 in the formation of active excitatory synapses. The first one has shown that inhibition of miR-

134 reduces spine density in hippocampal pyramidal neurons *in vivo,* thereby protecting from epileptic seizure (Jimenez-Mateos et al. 2012). The second one, focused on the role of mir-134 in GABAergic interneurons whereas all previous studies were conducted in excitatory neurons, demonstrate that miR134 downregulates DHHC9, the palmitoyltransferase of the regulatory GTPase H-Ras. To reconcile these contrasting results, the authors propose that miR-134 may exert its function on excitatory neurons indirectly, through the associated interneurons (Chai et al. 2013). The action of another miRNA has been also related to palmitoylation, a post-translational modification that is commonly used to mediate activity-dependent changes in synapses (Kang et al. 2008). MiR-138 is present at the post-synapse where it negatively regulates dendritic spine morphology through translational inhibition of the de-palmitoylating enzyme acyl protein thioesterase 1 (Apt1), an enzyme regulating the palmitoylation status of proteins with important function at the synapse like the α_{13} subunits of G proteins. Surprisingly, this increased volume was accompanied by decreased mEPSC amplitude that the authors ascribed to a decrease in GluR2 positive clusters found on the dendritic spines (Siegel et al. 2009). Another negative regulator of synaptic function is the miR-29a/b, which was identified in a screen for miRNAs involved in drugs of abuse-related plasticity (Lippi et al. 2011). In addition to regulating the structural aspects of dendritic spines, a number of miRNAs have been shown to directly regulate components of the post-synaptic density. The synaptically enriched miR-181a can target the GluR2 subunit of the 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor (AMPAR) through a conserved binding site in its 3′UTR (Saba et al. 2012b). In fact, surface levels of GluR2 are reduced in neurons overexpressing miR-181a. A number of mRNAs encoding post-synaptic density proteins appear to be shared targets of miRNAs and the fragile-X mental retardation protein (FMRP). The FMRP negatively regulates mRNA translation by directly interacting with target mRNAs. A first study found that the FMRP-bound PSD-95 transcript is under the regulation of miR-125a (Muddashetty et al. 2011). Interference

with miR-125a induces increased PSD-95 and increased spine number. In a second study, the authors identified miRNAs enriched in FMRP-bound RNA immunoprecipitation experiments (Edbauer et al. 2010). Of these miRNAs, miR-125b and miR-132 had significant effects on structural and synaptic plasticity when overexpressed. While miR-125b overexpression led to longer and thinner spines and decreased amplitude of mEPSC, miR-132 overexpression led to the formation of short, thicker spines and increased mEPSC amplitude and frequency, demonstrating that miRNA regulation at the synapse is not only negative. Turning their focus to miR-125b, the authors identified the N-methyl-d-aspartate (NMDA) receptor 2A (NR2A) as a direct miR-125b target (Edbauer et al. 2010). Another important miRNA is the activity-induced miR-132 that seems to play an important role positively regulating neuronal morphology and cellular excitability (Vo et al. 2005, Remenyi et al. 2010).

MiR-132 is transcribed in tandem with miR-212 sharing the same genic locus and primary transcript highly conserved among vertebrates (**Figure 8a**).

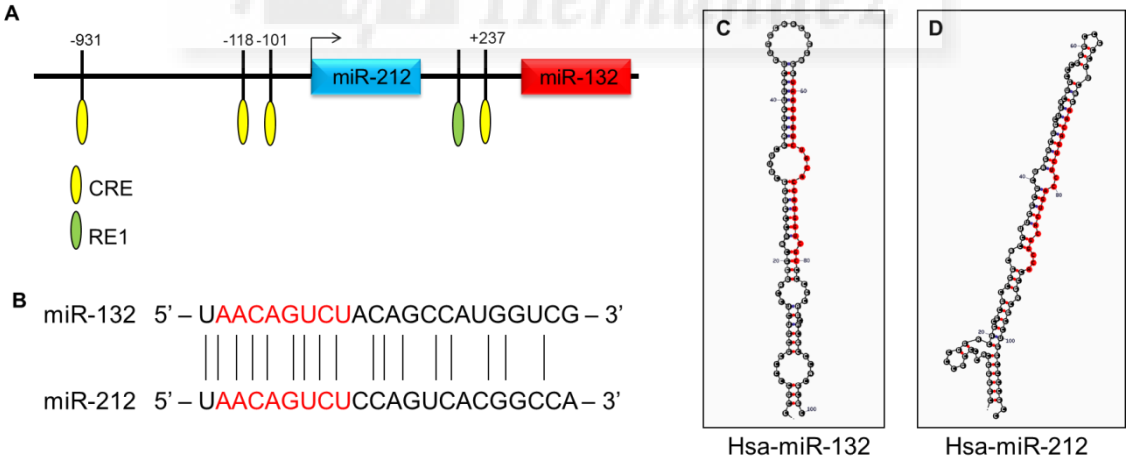

Figure 8. MiR-132 and miR-212 share the same genomic locus and seed sequence. A. miR132 and miR212 are transcribed from the same genic locus producing an unique primary transcript.Their transcription is under the control of CREB (in neurons) and REST (in non-neuronal cells) due to the presence of 3 CREB binding element (CRE) and one REST binding site (RE1). **B.** miR-132 and miR-212 exhibit similar mature sequences and share the same seed region (in red). miR-132 (**C**) and miR-212 (**D**) hairpin precursor are shown. Mature miRNA sequence is represented in red.

MiR-132 and miR-212 were first reported to be transcribed from the first and stable intron of the non-coding transcript DQ223059, localized on chromosome 10 in rats, and from the first intron of the non-coding transcript AK006051, localized on chromosome 11 in mice. In human, miR-132 and miR-212 are found in an intergenic region located on chromosome 17p13.3. As shown in **Figure 8b,** miR-132 and miR-212 exhibit similar mature sequences and share the same seed region and, potentially, the same targets even if this "double-targeting" was only demonstrated for few mRNAs, like MeCP2 (Klein et al. 2007, Im et al. 2010, Wada et al. 2010, Alvarez-Saavedra et al. 2011). The transcription of these two miRNAs is, in neurons, under the control of CREB (Impey et al. 2004, Vo et al. 2005) while, in non-neuronal cells, depends on REST (Conaco et al. 2006). In particular, one REST and several CREB-binding sites are conserved among mammals in the vicinity of miR-212/132 locus, suggesting an evolutionary conserved involvement of CREB and REST in the control of miR-212/132 expression. CREB is known to be involved in neuronal survival, maturation, differentiation and function, but also to control developmental plasticity, memory formation, adaptive behaviour, drug addiction and to regulate circadian rhythms (Lonze and Ginty 2002, Benito and Barco 2010). REST, on the other hand, is a transcriptional repressor known to actively repress neuronal gene expression in non-neuronal cells (Qureshi and Mehler 2009). Although only two transcription factors, REST and CREB, have been formally demonstrated to control the transcription of miR-212/132, other yet unidentified transcriptional regulators must be involved. The up-regulation of miR-132 in both cultured cortical and hippocampal neurons results in an increased dendritic outgrowth in an activity-dependent manner via suppression of a GTPase-activating protein p250GAP translation resulting in activation of the Rac1-PAK actin-remodelling pathway (Vo et al. 2005, Wayman et al. 2008, Impey et al. 2010). Beside its interaction with p250GAP mRNA, miR-132 also modulates dendritic plasticity by controlling the expression of another target, methyl CpG-binding protein 2 (MeCP2) (Klein et al. 2007). While a decrease in MeCP2 expression during the post-natal period postpones

neuronal maturation and synapses formation (Fukuda et al. 2005), its over-expression triggers dendrite and axon arborization (Jugloff et al. 2005), which suggests the need of maintaining MeCP2 levels between narrow ranges to ensure a proper neuronal development (Klein et al. 2007). Interestingly, as MeCP2 was suggested to control BDNF expression, which itself leads to the induction of miR-212/132 expression, miR-132 would take part in a feedback mechanism involved in the homeostatic control of MeCP2 expression (Klein et al. 2007). In agreement with these studies, over-expression of miR-132 in hippocampal neurons results in stubby and mushroom-shaped spines with an increase in average protrusion width strengthening synaptic transmission (Edbauer et al. 2010). Evidence emerging from *in vivo* studies has demonstrated that miR-132 effects on neuron morphogenesis are not limited to *in vitro* cultured cells. Indeed, the deletion of miR-132/miR-212 locus, whose predominantly active product is miR-132, is associated with a decrease in spine density and in dendrite length and arborization of newborn neurons in the mice adult hippocampus (Magill et al. 2010). The reported positive effect of miR-132 expression on dendritic growth and arborization of neurons may appear in contradiction with the observation that MeCP2 over-expression also results in dendritic and axonal arborization (Jugloff et al. 2005), being MeCP2 a miR-132 target. These conflicting results could be explained by the existence of several targets of miR-132 involved both in stimulating (such as MeCP2) or inhibiting neurite outgrowth and arborisation (such as p250GAP). In addition, the participation of still unidentified miR-132 targets in the positive or negative control of neuron arborization cannot be excluded. Initial *in vivo* experiments examined miR-132 expression in response to increased activity demonstrating that this miRNA is rapidly transcribed in hippocampus following enhanced neuronal activity but also after contextual fear conditioning task (Nudelman et al. 2010). Knockdown of miR-132 activity in newborn neurons in the adult hippocampus leads a reduction in spine density accompanied by decreased frequency, but not amplitude, of mEPSCs resulting in an impaired integration of these neurons in the hippocampal circuitry (Luikart et al. 2011).

On the other hand, *in vivo* over-expression experiments demonstrated that precise levels of miR-132 are required to maintain its functional role in plasticity. Indeed, relatively low levels of transgenic miR-132 expression (~1.5-fold), paralleling the level of expression in the hippocampus following a spatial memory task, significantly enhanced cognitive capacity without altering spine density (Hansen et al. 2013), whereas supra-physiological levels of miR-132 (>3-fold) inhibited learning increasing spine density in a reversible manner (Hansen et al. 2013). Another two studies, indicating the importance of a regulated balance in miR-132 levels, examined the role of this miRNA in the developmental refinement of neural circuits using as a model the formation of ocular dominancy (Mellios et al. 2011, Tognini et al. 2011). The other product of the miR-132/212 locus, miR-212, seems to be involved in the regulation of homeostatic plasticity lead to adaptive behaviors occurring in response to chronic drug exposure and drug addiction. In particular, miR-212 seems to play an important role to oppose the loss of control toward drug consumption. Upon chronic cocaine exposure miR-212 and, in a smaller extent, its cluster neighbour miR-132 is over-expressed in the dorsal striatum (Hollander et al. 2010). Under extended access to cocaine gain- and loss-of-function experiments showed that miR-212 interferes with the self-administered dose suggesting the involvement of this miRNA in the dampening of plasticity induced by chronic cocaine exposure, which causes the compulsive behavior. At the molecular level, striatal miR-212 decreases responsiveness to the motivational properties of cocaine by markedly amplifying the stimulatory effects of the drug on CREB signalling. This action occurs through miR-212-enhanced Raf1 activity, due to the inhibition of an unidentified repressor of Raf1 that is itself an activator of CREB, resulting in adenylyl cyclase sensitization and increased expression of the essential CREB co-activator TORC (Hollander et al. 2010). Moreover, miR-212 has been shown to target striatal MePC2 providing a parallel pathway accounting for the anti-addictive role of this miRNA toward cocaine (Im et al. 2010). Interestingly, striatal MeCP2, whose expression is increased in rats with extended access to cocaine, inhibits miR-212

expression that, in turn, represses MeCP2 expression. As MeCP2 levels are themselves correlated to BDNF expression, that controls cocaine intake, miR-212 would fine-tune the responses to drug abuse both by increasing CREB signalling and decreasing BDNF expression, resulting in a limited cocaine intake (Im et al. 2010).

2.4 Conditional Dicer Knockout mice as a model system for investigating miRNAs function in the brain

Knockout (KO) mice represent a powerful strategy to investigate specific function of a gene and/or a protein. In the case of Dicer, a conventional knockout was first generated to induce a system wide ablation of this gene and the consequent elimination of miRNAs by interfering with their biogenesis (Bernstein et al. 2003). This ablation resulted in early embryonic lethality, with Dicer-null embryos that arrest at embryonic day 7.5 and lack primitive streak markers, suggesting that Dicer is also essential for stem cell maintenance. To bypass the embryonic lethality and investigate the role of miRNA system disruption in specific brain regions and neuronal and non-neuronal cell types, a variety of conditional Dicer knockout mouse strains have been generated. In addition, the Cre-mediated recombination systems could add ulterior complexity to the studies allowing a temporal control on Dicer ablation by the use of specific promoters and/or by pharmacological induction of Dicer recombination, making possible to dissect the role of miRNAs in specific developmental stages or in adults. Conversely to the loss of individual specific miRNA, the global loss of the whole miRNA system in the brain is often associated with neurodegeneration (Gascon and Gao 2012). Several studies have shown that during early development the deletion of Dicer in the neural crest (NC) lineage using Wnt-1Cre-mediated recombination leads to the cell loss in enteric, sensory and sympathetic nervous systems but also malformations in midbrain and cerebellum (Huang et al. 2010, Zehir et al. 2010). The inactivation of Dicer in Purkinje neurons using Pcp-2Cre results in a slow progressive cerebellar

neurodegeneration and development of ataxia between 13 and 17 weeks of age (Schaefer et al. 2007). The loss of Dicer function in dopaminergic neurons using a Cre-mediated recombination under the control of dopamine transporter regulatory sequences (DAT) increases apoptosis and neurodegeneration in midbrain (Kim et al. 2007). On the contrary, the ablation of Dicer in post-mitotic dopaminergic neurons under the regulation of the dopaminergic receptor 1 (DR-1) is associated with ataxia, front and hind clasping, reduced brain size, smaller neurons and reduced lifespan to 10-12 weeks in absence of neurodegeneration (Cuellar et al. 2008). Similarly, Dicer loss in mature olfactory neurons in mouse has no apparent consequence over several months (Choi et al. 2008) whereas the loss of Dicer in retina leads to progressive degeneration of tissue architecture (Damiani et al. 2008, Pinter and Hindges 2010, Papadopoulou et al. 2012).

Many studies have been performed to elucidate the role of miRNAs in forebrain using different temporal windows for Dicer function disruption. Many studies focused on the role of miRNA system in cortex development. The ablation of Dicer in the neocortex in the early embryonic stage under the control of Emx-1Cre system, that starts to be expressed around the embryonic day (E) ~9.5, results in massive hypotrophy of the postnatal cortex, defective cortical layering postnatally, and death of the mice shortly after weaning probably due to the death of neuronal progenitors (De Pietri Tonelli et al. 2008, Kawase-Koga et al. 2009). During the late embryonic stage cortical-specific NestinCre-mediated Dicer recombination also results in strong cortical defects but caused to altered migration of late-born neurons (Kawase-Koga et al. 2009). Interestingly, Nestin-Cre line produced very few Dicer conditional knockout embryos (6.8% at embryonic day 18.5) with no surviving newborns whereas Dicer knockout embryos from the Emx1Cre line were collected at Mendelian ratios (24.8%) and they could survive until postnatal day 30 (P30) (Kawase-Koga et al. 2009). Blocking miRNA biogenesis in post-mitotic cortical neuron in the mouse cortex at perinatal stages does

not significantly affect neurogenesis, neuronal survival, and layer organization (Hong et al. 2013). However, the neuronal packing density is greatly increased in the cortical plate, resulting in a significantly reduced cortical size, and neurite outgrowth and soma size development are significantly reduced in cultured Dicer KO neurons (Hong et al. 2013). The importance of miRNAs for the correct development of the hippocampus was also demonstrated (Li et al. 2011). In fact, conditional ablation of Dicer at different time points results in abnormal hippocampal morphology and altered number of hippocampal progenitors due to altered proliferation and increased apoptosis but, if the lack of miRNAs at earlier stages causes altered differentiation of hippocampal neurons in the CA1 and DG regions, the lack of these small RNAs at a later stage specifically affects neuronal production in the CA3 region (Li et al. 2011).

In order to investigate the role of Dicer and miRNAs only in excitatory forebrain neurons *in vivo*, Dicer floxed mice expressing an alpha-calmodulin kinase II (CAMKII-α) Cre were used to selectively inactivate Dicer in these neurons (Davis et al. 2008). Inactivation of Dicer in forebrain neurons results in an array of phenotypes including microcephaly in absence of lamination defects, likely caused by a 5.5-fold increase in early postnatal apoptosis, reduced dendritic branch elaboration and large increase in dendritic spine length with no concomitant change in spine density (Davis et al. 2008). Interestingly, when Dicer ablation was triggered in fully developed adult mice in a tamoxifen-inducible forebrain-restricted KO strain, a slow neurodegenerative process was preceded by enhanced memory strength, higher efficacy at CA3-to-CA1 synapses and the presence of elongated filopodia-like shaped dendritic spines in CA1 pyramidal neurons (Konopka et al. 2010). In the hypothalamus, conditional Dicer ablation in POMC neurons, a neuronal population specifically located in the arcuate nucleus (ARC), is associated with neurodegeneration and dysfunction leading to obesity (Schneeberger et al. 2012, Greenman et al. 2013). In contrast, adult deletion of Dicer in the ARC caused hyperphagia and obesity but not neuronal cell death (Vinnikov et al.

2014, Fiorenza et al. 2015, Mang et al. 2015). Dicer function is also found to be important in the regulation of oligodendrocytes differentiation (Shin et al. 2009, Dugas et al. 2010, Zhao et al. 2010).

Loss of Dicer in Schwann cells arrests their differentiation and the myelination process resulting in early lethality (Bremer et al. 2010, Yun et al. 2010), alters myelin-related gene expression (Pereira et al. 2010), cause a severe neurological phenotype resembling congenital hypomyelination (Yun et al. 2010) and affect peripheral nerve regeneration (Wu et al. 2012). Finally, conditional ablation of Dicer in astrocytes, controlled by a mouse Gfap gene regulatory sequence, leads to abnormal astrocytic maturation, maintenance and function that cause non-cell-autonomous neuronal dysfunction resulting in ataxia, severe progressive cerebellar degeneration, seizures, uncontrollable movements, and premature death by postnatal week 9-10 (Tao et al. 2011).

3. The miRNA system and brain disorders

Dysregulation of miRNA expression and function and/or alteration of enzymatic function involved in miRNA pathway have been implicated in the pathogenesis of a variety of neuropsychiatric, neurodevelopmental and neurodegenerative disorders, including neurodevelopmental and psychiatric disorders, such as Rett syndrome (Urdinguio et al. 2010, Wu et al. 2010), Fragile-X-syndrome (Moreau et al. 2011, Fass et al. 2014, Sun and Shi 2014b, Wang et al. 2014b, Bavamian et al. 2015), autism (Abu-Elneel et al. 2008, Ghahramani Seno et al. 2011, Mellios and Sur 2012) and bipolar disorder (Moreau et al. 2011, Fass et al. 2014, Sun and Shi 2014b, Wang et al. 2014b, Bavamian et al. 2015). We discuss in the next section in greater details some relevant examples.

Importantly, the study of the role of miRNAs in both physiological and pathological conditions is not only important for understanding the molecular mechanisms underlying pathogenic alterations but also for the development of novel therapeutic approaches.

3.1 Schizophrenia

Schizophrenia is a devastating neuropsychiatric disorder of neurodevelopmental origin, arising through complex interplay between genetic and epigenetic factors (Harrison 1997) and characterized by a variety of symptoms including hallucinations, delusions and disruption of logical thought patterns, affective flattening and depression (Sun and Shi 2014a). Interestingly, patients with 22q11.2 deletion syndrome (22q11.2DS), also known as the velocardiofacial/DiGeorge syndrome, a phenotypically heterogenous disease which is caused by a hemizygous microdeletion on the long arm of chromosome 22 in the region q11.2, showed a 30-fold increase in the risk of schizophrenia (Forstner et al. 2013). Importantly, the characterization of an animal model carrying the 22q11.2 microdeletion showed alterations in the biogenesis of brain miRNAs (Stark et al. 2008). Primary candidate genes in the region are the DiGeorge syndrome critical region gene 8 (DGCR8), which encodes a component of the microprocessor complex essential for miRNA biogenesis, and the MIR185 gene, which encodes miR-185 (Karayiorgou et al. 2010). Both genes are located within the minimal 1.5 Mb deletion region at 22q11.2 (Karayiorgou et al. 2010). A recent human study confirmed a down-regulation of MIR185 expression to 0.4-fold normal levels in the peripheral blood of patients with 22q11.2DS suggesting that pronounced miR-185 down-regulation also occurs in patients with 22q11.2DS (de la Morena et al. 2013). In addition, the involvement of miRNA-dependent dysregulation in idiopathic schizophrenia is supported by the results of a large Genome-Wide Association Study (GWAS) that reveals the strong association between schizophrenia and a single-

nucleotide polymorphism (SNP) in an intron of the putative primary transcript for miR-137 (Consortium 2011), being miR-137 a known regulator of neurodevelopment.

Several studies have analysed miRNA levels in post-mortem brains from schizophrenia patients and identified multiple miRNAs that are differentially expressed. Thus, initial studies on post-mortem brains revealed an overall decrease in miRNA expression in the prefrontal cortex of schizophrenia patients (Perkins et al. 2007). A subsequent independent study showed that the expression of miR-132 and miR-132* was significantly decreased in brains of schizophrenia patients (Miller et al. 2012). Given that miR-132 has been shown to potentiate NMDA receptor depolarization (Cheng et al. 2007), the reduced expression of miR-132 could be responsible for the hypo-function of the NMDA receptor in schizophrenia patients. Later studies reported an increase in the expression of a set of miRNAs in medial temporal regions of schizophrenia patients coinciding with elevated expression of components in the miRNA biogenesis machinery (Beveridge et al. 2008, Beveridge et al. 2010, Santarelli et al. 2011).

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3.2 Neurodegenerative diseases

MiRNAs also seem to play an important role in neurodegenerative diseases, such as Alzheimer's disease (AD) (Lukiw 2007, Hebert et al. 2008, Wang et al. 2008, Nelson and Wang 2010, Nunez-Iglesias et al. 2010, Shioya et al. 2010, Wang et al. 2011, Long et al. 2012, Hebert et al. 2013, Banzhaf-Strathmann et al. 2014, Chang et al. 2014, Cui et al. 2014, Garza-Manero et al. 2015), Parkinson's disease (PD) (Kim et al. 2007, Harraz et al. 2011, Minones-Moyano et al. 2011, Vallelunga et al. 2014), Huntington's disease (HD) (Johnson et al. 2008, Packer et al. 2008, Johnson and Buckley 2009, Gaughwin et al. 2011, Ghose et al. 2011, Lee et al. 2011, Jin et al. 2012, Cheng et al. 2013, Goodall et al. 2013, Kozlowska et al. 2013, Kocerha et al. 2014) and prion

diseases (Saba et al. 2008, Montag et al. 2009, Bellingham et al. 2012, Gibbings et al. 2012, Montag et al. 2012, Saba et al. 2012a). All these conditions are characterized by the progressive loss of specific neuronal groups in the central nervous system, resulting in significant motor and cognitive disability, and have been associated with altered expression of certain miRNAs that target relevant proteins for pathogenesis and/or to the presence of polymorphisms in miRNA coding genes or in specific miRNA target sites.

In the case of HD, an autosomal dominant inherited disorder caused by an elongated CAG repeat expansion in the huntingtin (HTT) gene and characterized by severe degeneration of the corpus striatum and atrophy of several brain regions, including the caudate nucleus, putamen and globus pallidus and cortex, strong evidence suggests that the widespread gene expression changes detected in this condition can be, at least, partially attributed to miRNA dysregulation (Seredenina and Luthi-Carter 2012). The HTT protein directly interacts with Ago2 and is found to localize into P bodies. Depletion of wild type HTT compromises miRNA mediated gene silencing and the mutant protein disrupts neuronal P body integrity (Savas et al. 2008). Other evidences suggest that other key components of miRNA biogenesis are dysregulated in mouse models of the disease, including Dicer, Drosha and Exportin-5, at different stages of the disease course (Lee et al. 2011). An alternative mechanism to explain the aberrant gene expression profile observed in HD is based on the increased nuclear localization of REST. As previously mentioned, REST is a transcriptional repressor that acts to silence neuronal gene expression in non-neuronal cells. In healthy neurons REST is sequestered in the cytoplasm, but in Huntington's disease there is increased nuclear translocation of REST in neurons leading to increased gene repression, which has a negative effect on survival (Zuccato et al. 2007). In addition to targeting mRNA, REST has been shown to regulate miRNAs, including a neuronal miRNA family containing miR-124a, miR-132, miR-9, and miR-9* (Conaco et al. 2006,

Johnson et al. 2008, Marti et al. 2010). The majority of REST-regulated miRNAs identified to date have displayed reduced expression in Huntington's patient brain tissue and models of the disease.

3.3 Epilepsy

MiRNAs regulation has been also related to different types of pathological conditions characterized by neuronal excitotoxicity and over-excitation (Eacker et al. 2013), such as stroke and epilepsy. Epilepsy is a chronic neurologic disorder characterized by recurrent and unprovoked seizures resulting of abnormal, synchronous discharges of groups of neurons in the brain and affecting more than 50 million of people worldwide. Epileptogenesis is associated with several factors, including complex temporal and spatial abnormalities of neural network structure, activity mediated by posttranslational modifications of proteins, activation of immediate early genes (IEGs) and other alterations in profiles of gene expression and function, like GABAA receptor subunit, CREB, JAK-STAT, BDNF and EGR3 (Li et al. 2013). Focal epilepsies result in seizures beginning in a localized fashion, which then spread by recruitment of other brain areas due to focal pathological changes. Therefore, the occurrence of epileptic activity is probably caused by greater spread and neuronal recruitment secondary to a combination of enhanced connectivity and excitatory transmission (Duncan et al. 2006, Li et al. 2013). Temporal lobe epilepsy (TLE) is the most common syndrome in adults and is thought to involve neuronal death or dysfunction, changes in ion channel function, gliosis, neuroinflammation and neurogenesis (McNamara et al. 2006, Pitkanen and Lukasiuk 2011).

Emerging evidence shows that epilepsy and epileptogenesis are controlled by epigenetic factors and gene products that regulate multiple genes and proteins at system level (Kobow and Blumcke 2011, Lubin 2012). In this context, researchers have

turned to the question of whether miRNAs might be altered by seizures, during both epileptogenesis and chronic epilepsy. Recent profiling data show that a subset of miRNAs seems to be specifically altered in the epileptic brain. These “epilepsy – specific” miRNAs include miR-146a, miR-34a, miR-134, miR-132 and miR-21 (Li et al. 2013), which is consistent with the regulation of the expression of some of these miRNAs by neuronal activity discussed in previous sections. Genome-wide miRNA expression profile studies in human TLE revealed important insights into the role of these small RNA in human epilepsy. In one study, 20 human hippocampi including both sclerotic and non-sclerotic samples were used (Kan et al. 2012). 51 miRNAs with >2-fold changes are found displaying up- as well as down-regulation. Interestingly, several other features of miRNA expression in human TLE are observed, including the reduction of several miRNAs levels in neurons and the increase of the same miRNAs in glia, and the nuclear localization of a number miRNAs that are generally located in cytoplasm (Kan et al. 2012). These two observations could indicate novel function and/or failure of a part of the miRNA biogenesis pathway, such as maturation or export. In another study, the strong miRNA down-regulation in sclerotic hippocampi from human TLE patients was confirmed (McKiernan et al. 2012a) suggesting a failure in the miRNA biogenesis pathway. The potential cause of this reduction was proposed to be the loss of Dicer expression that results strongly reduced in these samples without changes of other biogenesis components (McKiernan et al. 2012a).

Seizures could be induced in rodents by injection of pilocarpine, a muscarinic acetylcholine receptor agonist, or injection of kainic acid, an agonist of the kainate-type glutamate receptor. Injection of either of these compounds results in establishment of status epilepticus (SE) in rodents mimicking the human TLE (Jimenez-Mateos and Henshall 2013). In rats, pilocarpine injection induces SE and results in increased levels of mature miR-132 and its primary transcript (Nudelman et al. 2010). Shortly after, another group profiles miRNAs expression changes after kainic-induced seizure

concluding that a strong association existed between miRNAs and mRNAs profiles, in particular, for processes like cell death, immunological response and gene expression (Liu et al. 2010). All these finding suggest that miRNA levels changes can be functionally relevant in epilepsy. After these two first reports, numerous miRNA profiling studies have been produced using a variety of experimental models of status epilepticus, including systemic kainic acid in rats (Liu et al. 2010, Pichardo-Casas et al. 2012), systemic kainic acid in mice (McKiernan et al. 2012d), intra-amygdala kainic acid in mice (Jimenez-Mateos et al. 2011) and pilocarpine in rats (Hu et al. 2011, Hu et al. 2012). Together, these studies reveal the existence of a number of potentially conserved molecular signatures of miRNA changes after seizures (Jimenez-Mateos and Henshall 2013). These include up-regulation of the pro-apoptotic p53-induced miRNA miR-34a (Hu et al. 2011, Hu et al. 2012, Sano et al. 2012); miR-132 (Nudelman et al. 2010, Hu et al. 2011, Jimenez-Mateos et al. 2011), which has anti-inflammatory effect; miR-134 (Jimenez-Mateos et al. 2012, Peng et al. 2013, Wang et al. 2014a), whose inhibition by antagomirs is actually studied like potential therapeutic tool; miR-146a, (Aronica et al. 2010, Iyer et al. 2012, Omran et al. 2012), miR-21 (Peng et al. 2013), miR-199a and miR-375 (Hu et al. 2011, Jimenez-Mateos et al. 2011).

3.4 Obesity and feeding disorders

The obesity epidemic has become a global issue (Finucane et al. 2011), causing major human and economic consequences. Obesity is associated to high rates of morbidity and mortality due to a major risk to develop serious and chronic conditions, such as type-2 diabetes, cardiovascular diseases, hypertension, stroke, and certain forms of cancer (Kopelman 2000). The global epidemic of obesity results from a combination of genetic susceptibility, increased availability of high-energy foods and decreased requirement for physical activity in modern society. However, and despite the

magnitude of this public health problem, effective and safe pharmacological strategies are currently unavailable (Dietrich and Horvath 2012).

Obesity is generally the result of a positive energy balance, in which the caloric intake exceed the body energy expenditure and the excess of calories is stored like fat. Generally, there are no genetic defects leading to this condition and the primary cause is the hyperphagia. Although monogenic causes of morbid obesity are uncommon, several obesity-related genetic disorders have been identified in humans.

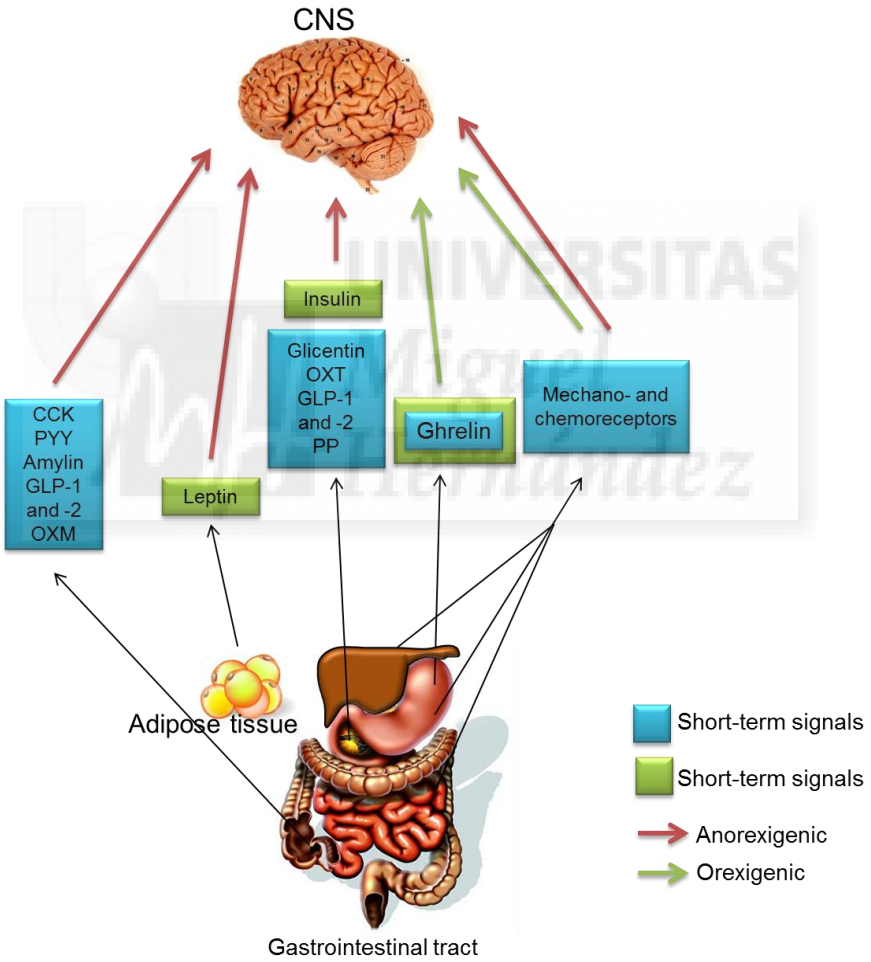

Figure 9. Peripheral signals conveying to the CNS for regulating food intake. The gastrointestinal tract produces several polypeptides able to act centrally for regulating food intake and body weight. Importantly, except for ghrelin that is secreted during starvation and acts inducing food intake, almost all the peptides known to the date act like anorexigenic signals. Short-term signals are produced acutely in response to ingestion and control the size of individual meal, whereas long-term signals, like leptin and insulin, are produced in response to food intake and proportionally to the fat store and are responsible to monitor and transmit to the CNS information about the amount of nutrient reservoir of the body.

Obesity is accompanied by hyperphagia in several classical but rare genetic obesity-related syndromes, such as Prader–Willi syndrome (PWS) and Alström syndrome (ALMS). Consequently, understanding the precise mechanisms implicated in the regulation of food intake and energy balance is crucial for the development of more effective anti-obesity therapeutical approaches. Reflecting the fundamental biological importance of adequate nutrient supply and energy balance, food intake and energy expenditure are controlled by complex circuits involving different brain regions, such as the hypothalamus and brain stem, and a variety of signals that, originated peripherally, are able to conveying metabolic information to the nervous system (**Figure 9**). All these circulating factors could act peripherally on the gastrointestinal tract, directly or by vagal fibers, or centrally on specific brain regions able to detect and interpret the information about the metabolic status of the organism and induce behavioral responses. In recent years, hypothalamus emerged like the critical area of the central nervous system (CNS) regulating energy homeostasis. This hypothalamic system includes several anatomically well-defined nuclei, including the arcuate nucleus (ARC), the ventromedial (VMH), dorsomedial (DMH), lateral (LH) and paraventricular (PVN) nuclei of hypothalamus. These nuclei have reciprocal synaptic connections and have been extensively studied for their roles in homeostatic regulation of appetite, food intake and body weight. Its position, adjacent to the median evidence, a circumventricular organ with fenestrated capillaries and hence an incomplete BBB (Peruzzo et al. 2000) that exposes ARC to circulating factors, and its projection to both intra-hypothalamic (e.g. VMH, DMH, LH and PVN) and extra-hypothalamic targets, make ARC the key player in the regulation of food intake and energy homeostasis (**Figure 10**).

The ARC contains two populations of neurons with opposing effects on food intake (Bewick et al. 2005). Orexigenic neurons co-expressing neuropeptide Y (NPY)

and agouti-related protein (AgRP) are medially located in the ARC (Hahn et al. 1998, Bewick et al. 2005).

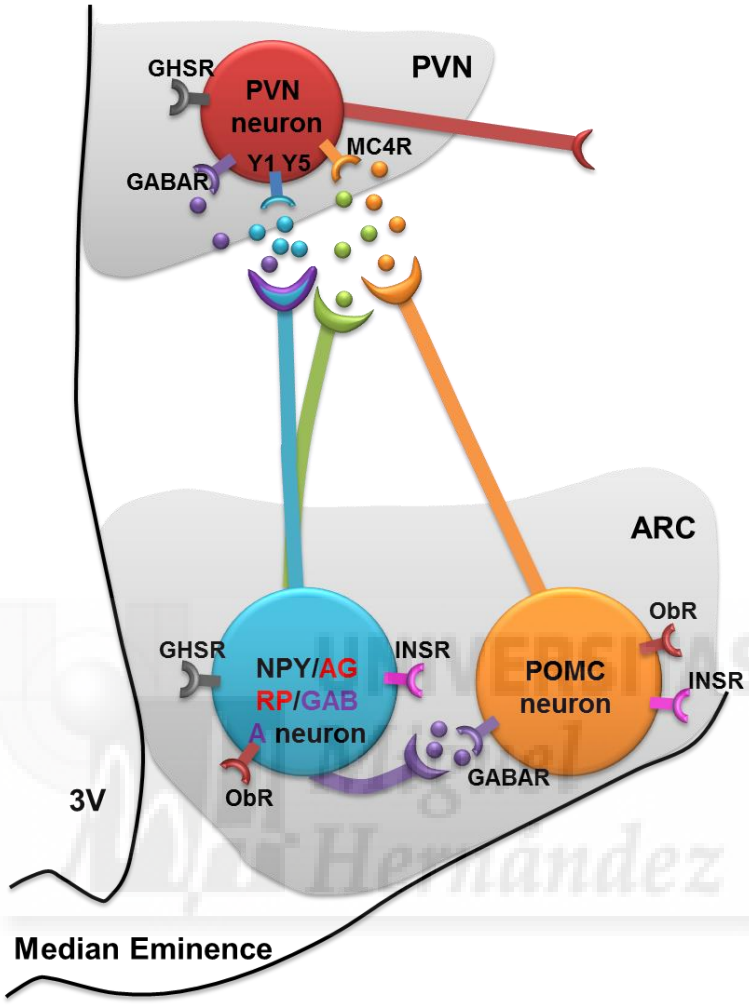

Figure 10. Hypothalamic ARC-PVN circuits controlling food intake and body weight regulation. Arcuate nucleus (ARC) contains two anatomically and functionally different neuronal population connected between them and with the paraventricular nucleus (PVN) (in red) forming a circuit named melanocortin system. NPY (in blue), Agrp (in red) and GABA (in purple) are coexpressed in orexigenic neurons that, when activated by ghrelin, stimulate food intake. The second ARC neuronal population expressing POMC (in orange) produce the anorexigenic α-MSH. NPY acts in PVN on Y_1 and Y_5 receptors to induce food intake whereas Agrp antagonized MCR4 preventing the orexigenic effect of α-MSH. GABA proyections fron NPY/Agrp neurons reach POMC neurons forming an unidirectional feedback control loop. PVN proyects to many area in the brain to control feeding behavior, energy expenditure and adiposity. Both NPY and POMC neurons respond to circulating hormones and contain several receptors, such as for leptin (ObR), insulin (INSR). Importantly, only NPY neurons contain receptor for ghrelin (GHSR). PVN neurons also contan GHSR but its function is actually u nknown. 3V, third ventricle. More details in the text. Adapted from **Andrews 2011**.

On the other hand, anorexigenic neurons in the lateral ARC express α-melanocyte-stimulating hormone (α-MSH) derived from pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) (Elias et al. 1998). The axons of these neurons project to "second-order" neurons, located in the PVN, secreting anorexigenic molecules like thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and oxytocin, and in LH, where orexigenic peptides, like melanin-concentrating hormone (MCH) and orexin, are produced. The balance between the activities of these neuronal circuits is critical to body weight and appetite regulation. Importantly, the existence of an unidirectional connection between these two neuronal populations in the ARC allows an internal feedback control (Horvath et al. 1992). In fact, the orexigenic NPY/AgRP neurons projects to POMC/CART neurons inhibiting their anorexigenic function, thus promoting feeding. NPY/AgRP neurons activity is regulated by the nutritional signals and higher in the fasting state (Yang et al. 2011b, Liu et al. 2012). Two of these signals, ghrelin and leptin, are the major players in the regulation of NPY/AgRP neuronal function with opposite effects. Indeed, whereas leptin inhibits the orexigenic activity of these neurons, ghrelin is able, by the binding to its receptor present in the surface of NPY/AgRP neurons, to induce a strong activation of their function that results in the stimulation of orexigenic nuclei, such as LH, and in the inhibition of POMC/CART neuronal activity. Importantly, ghrelin is the unique orexigenic peptide known to the date and is approximately equipotent to NPY (Asakawa et al. 2001, Wren et al. 2001).

Initial studies reported that the rodent hypothalamus is differentially enriched in specific miRNAs such as miR-124a, miR-125a, miR-136, miR-138, miR-212, miR-338, miR-451, let-7c genes and particularly miR-7a and miR-7b (Farh et al. 2005, Bak et al. 2008, Schneeberger et al. 2015). More specifically, miRNA expression profiling of the ARC and paraventricular nucleus (PVN) of the rat hypothalamus has revealed similar expression patterns from a set of >210 miRNA genes with specific enrichment of ~20

miRNAs, including seven of the eight genes of the let-7 family, miR-9, miR-30, and the two miR-7 genes (Amar et al. 2012). Consistently, in situ hybridization studies aimed at establishing the cellular localization of miR-7a and miR-7b, showed a more restricted expression pattern for the former being mainly located in the ARC (Herzer et al. 2012). Specifically, miR-7a is preferentially expressed in AgRP neurons rather than POMC neurons (Herzer et al. 2012) suggesting specific pathophysiological functions for miRNAs upon energy balance control. The identity of particular hypothalamic miRNAs implicated in systemic energy balance control just started to be unveiled. In an attempt to describe relevant hypothalamic miRNAs, recent studies have profiled miRNA expression in the context of metabolic distress (Schneeberger et al. 2015). Investigating differential miRNA expression patterns in the hypothalamus from leptin-deficient ob/ob mice versus controls 11 out of 524 miRNAs have found significantly modified with a fold-change >2 in ob/ob mice: 10 were up-regulated and 1 down-regulated (Crepin et al. 2014). Increased expression was confirmed for only 3 miRNAs (miR-200a, miR-200b and miR-429), and their expression was normalized after leptin treatment. miR-200a was also increased in the hypothalamus of leptin receptor-deficient db/db mice (Crepin et al. 2014). Importantly, additional studies indicated that Insulin receptor 2 (Irs-2) and leptin receptor are direct targets of miR-200a (Crepin et al. 2014). In a previous study, using a similar large-scale approach, the same authors reported that impairment of leptin action perinatally also caused disturbances in hypothalamic miRNAs expression. Administration of a leptin antagonist in newborn rats promoted overweight and leptin/insulin resistance as well as changes in hypothalamic miRNA expression profile in adulthood (Benoit et al. 2013). Interestingly, 38 miRNAs were found to be differentially expressed including miR-200a (Benoit et al. 2013) indicating that overexpression of miR-200a in obesity may interfere with insulin and leptin pathways in the hypothalamus by down-regulating key signalling mediators such as Irs-2 and leptin receptor respectively. Another study assessed the effects of short and long-term nutritional manipulations on hypothalamic miRNA expression in rats

(Sangiao-Alvarellos et al. 2014). Chronic calorie restriction (CR) or high-fat diet (HFD) administration altered the expression of 74 out of 641 miRNAs analysed, including let-7a, miR-9*, miR-30e, miR-132, miR-145, miR-200a, and miR-218 (Sangiao-Alvarellos et al. 2014). Algorithm-based target predictions included key components of signalling pathways found to be disturbed in obesity, such as NFKβ, interleukins, PI3K/AKT, ceramides, insulin receptor, p70S6K and JAK/STAT (Sangiao-Alvarellos et al. 2014). miR-103 has also been recently implicated in the hypothalamic control of energy homeostasis. Using a combination of hypothalamic miRNA profiling and *in silico* predictions, Vinnikov and collaborators found that bilateral injection of miR-103 mimic attenuated the obesogenic phenotype of mice lacking Dicer in forebrain neurons reducing their hyperphagia (Vinnikov et al. 2014). This effect was likely mediated by the ability of miR-103 to modulate hypothalamic PI3K-Akt-mTOR activity, key components of the insulin signalling pathway, although the precise target remains unknown (Vinnikov et al. 2014).

Finally, according with a role for miRNAs in food intake and energy metabolism regulation, hypothalamic Dicer expression is regulated by nutrient availability, pathophysiological conditions of nutrient excess and genetic obesity. Fasting up-regulates Dicer, while the expression of other transcripts implicated in miRNA biogenesis were unaltered. In contrast, genetic and induced rodent models of energy excess exhibited decreased expression of Dicer in the hypothalamus (Schneeberger et al. 2012). Importantly, a very recent study found alterations in miRNA levels in obese patients affected by Prader-Willi syndrome and Alström syndrome, two rare genetic obesity-related syndromes characterized by hyperphagia, suggesting a possible pathogenic role for these small RNAs in these conditions (Butler et al. 2015).

HIPOTHESIS AND OBJECTIVES

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Different approaches are being used to clarify the mechanisms of action and the physiological role of miRNAs. Although numerous studies have investigated the involvement of individual miRNAs in specific tissue, the role of miRNA system as a whole is far to be understood, especially in the context of a complex structure such as the brain. Unveiling the function of the miRNA system in the brain can be extremely important to develop new therapeutical approaches for different neuropsychiatric diseases in which global changes in miRNAs levels have been observed.

The conditional genetic ablation of Dicer in neurons represents a powerful model to investigate the role of miRNA system in brain function because of the critical involvement of this enzyme in miRNA biogenesis. However, previous investigations on Dicer conditional KO mice were constrained by ongoing neurodegeneration, which makes difficult the identification of phenotypes directly originated by the loss of the miRNA system. In order to study the role of the miRNA system in adult forebrain neurons avoiding the confounding effects derived from neurodegeneration and to identify possible miRNA-regulated processes, we characterized a strain of mice in which the exon 24 of *Dicer1* is selectively eliminated in forebrain principal neurons, leading to the production of a truncated inactive protein in these cells. Importantly, in this strain forebrain-restricted Cre-mediated recombination that can be induced by the experimenter at any time by tamoxifen administration.

We defined the following six objectives:

1. To perform a genomic screen for the detection of changes in small RNA species to identify reductions in specific miRNAs as a consequence of Dicer/miRNA system loss.

2. To perform a differential genomic screen for mRNAs to identify possible direct miRNA targets affected by Dicer loss.

3. To compare the results from the two complementary differential genomic screens for changes in small RNA species and mRNAs to identify specific molecules and biological processes affected by Dicer/miRNA system loss.

4. To explore the impact of impaired miRNA maturation in the regulation of hippocampal neuronal function and plasticity, as well as activity-driven gene expression using novelty exploration and kainate administration as stimulating paradigms.

5. To confirm the paradoxical memory enhancement previously observed in Dicer-ifKO mice and further explore this phenotype to likely add new insight.

6. To investigate the impact of disrupting miRNA biogenesis in feeding behavior.

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RESULTS

Blocking miRNA biogenesis in adult forebrain neurons enhances seizure susceptibility, fear memory and food intake by increasing neuronal responsiveness

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ORIGINAL ARTICLE

Blocking miRNA Biogenesis in Adult Forebrain Neurons Enhances Seizure Susceptibility, Fear Memory, and Food Intake by Increasing Neuronal Responsiveness

Anna Fiorenza, Jose P. Lopez-Atalaya, Victor Rovira, Marilyn Scandaglia, Emilio Geijo-Barrientos, and Angel Barco

Instituto de Neurociencias (Universidad Miguel Hernández-Consejo Superior de Investigaciones Científicas), Av. Santiago Ramón y Cajal s/n., 03550 Sant Joan d'Alacant, Alicante, Spain

Address correspondence to Dr Angel Barco. Email: abarco@umh.es

Abstract

The RNase Dicer is essential for the maturation of most microRNAs, a molecular system that plays an essential role in fine-tuning gene expression. To gain molecular insight into the role of Dicer and the microRNA system in brain function, we conducted 2 complementary RNA-seq screens in the hippocampus of inducible forebrain-restricted *Dicer1* mutants aimed at identifying the microRNAs primarily affected by Dicer loss and their targets, respectively. Functional genomics analyses predicted the main biological processes and phenotypes associated with impaired microRNA maturation, including categories related to microRNA biology, signal transduction, seizures, and synaptic transmission and plasticity. Consistent with these predictions, we found that, soon after recombination, Dicer-deficient mice exhibited an exaggerated seizure response, enhanced induction of immediate early genes in response to different stimuli, stronger and more stable fear memory, hyperphagia, and increased excitability of CA1 pyramidal neurons. In the long term, we also observed slow and progressive excitotoxic neurodegeneration. Overall, our results indicate that interfering with microRNA biogenesis causes an increase in neuronal responsiveness and disrupts homeostatic mechanisms that protect the neuron against overactivation, which may explain both the initial and late phenotypes associated with the loss of Dicer in excitatory neurons.

Key words: activity-driven transcription, Dicer, epilepsy, learning and memory, miRNAs, neuronal excitability, neuronal homeostasis, obesity, regulation of gene expression

Introduction

The *Dicer1* gene encodes the cytoplasmic RNase III Dicer that is essential for the production of mature microRNAs (miRNAs) (Bernstein et al. 2001). These molecules are approximately 22 nucleotide-long small noncoding RNAs that posttranscriptionally repress gene expression by base pairing to the complementary sequence in the 3′-untranslated region of target messenger RNAs (mRNAs), interfering with their translation and/or promoting their degradation (Nilsen 2007; Carthew and Sontheimer 2009; Inui et al. 2010). Although many studies have focused on binary miRNA–-target interactions, systems biology analyses indicate that miRNAs may act as molecular buffers that prevent undesirable fluctuations of protein levels providing robustness to biological systems (Hornstein and Shomron 2006; Li et al. 2009; Ebert and Sharp 2012; Pelaez and Carthew 2012). Transcription occurs in stochastic bursts (Raj et al. 2006) and miRNAs may contribute to reducing fluctuations of the target genes at the protein level (Ebert and Sharp 2012).

In line with this model, many gene knockout experiments have shown that the elimination of individual miRNAs frequently causes no gross phenotype or yields modest results despite of the large number of target genes potentially affected (Ebert and

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Sharp 2012). Additionally, interfering with miRNA biogenesis has more dramatic consequences during development than in fully differentiated cells. Thus, conventional knockout (KO) mice for Dicer show very early embryonic lethality (Bernstein et al. 2003), while the impact of *Dicer1* ablation in adult tissues, although consistently deleterious, is subtle and cell type-dependent (Harfe et al. 2005; Kawase-Koga et al. 2009; Georgi and Reh 2010; Zehir et al. 2010). Thus, the loss of Dicer in the thymic epithelium or retina leads to progressive degeneration of tissue architecture (Damiani et al. 2008; Pinter and Hindges 2010; Papadopoulou et al. 2012), whereas its loss in the mouse olfactory system has no apparent consequence over several months (Choi et al. 2008).

In the central nervous system, numerous studies have associated the lack of Dicer with slow and progressive neurodegeneration. For example, the loss of Dicer in either Purkinje neurons (Schaefer et al. 2007) or postnatal astroglia (Tao et al. 2011) caused ataxia and a slow cerebellar degeneration, whereas its loss in excitatory forebrain neurons in the postnatal brain was associated with microcephaly, reduced dendritic branching and also progressive neurodegeneration (Davis et al. 2008; Hebert et al. 2010; Li et al. 2011; Hong et al. 2013). In the case of Dicer loss in dopaminoceptive neurons, however, ataxia and decreased lifespan occurred in the absence of apparent neurodegeneration (Cuellar et al. 2008). Intriguingly, when gene ablation was triggered in fully developed adult tamoxifen-inducible forebrain-restricted KO mice, the slow neurodegenerative process was preceded by enhanced memory strength, higher efficacy at CA3-to-CA1 synapses and the presence of elongated filopodia-like shaped dendritic spines in CA1 pyramidal neurons (Konopka et al. 2010). The molecular mechanism of the dual impact of Dicer loss in adult forebrain excitatory neurons remains unknown.

To identify candidate mechanisms that could explain these phenotypes, we conducted 2 complementary genomic screens in the hippocampus of inducible forebrain-restricted *Dicer1* mutants aimed at identifying the main miRNAs affected and their targets. These screens revealed the specific impact of Dicer depletion on miRNA biogenesis and identified a number of miRNAs that are particularly sensitive to the loss of Dicer in neurons. Notably, a significant proportion of the mRNAs upregulated in mutant mice were predicted targets of the deregulated miRNAs. Further characterization of these animals revealed the rapid emergence of a number of phenotypes after *Dicer1* ablation that are highly consistent with the results of functional genomics analyses, such as an exacerbated seizure response, enhanced induction of immediate early genes (IEGs), memory improvement, hyperphagia, and increased neuronal excitability. Overall, our results contribute to explaining both the initial and late consequences of interfering with Dicer function in excitatory neurons and indicate that Dicer and the miRNA system play a critical role regulating neuronal responsiveness and homeostasis.

Results

Inducible Forebrain-Restricted Dicer1 Ablation Triggers Slow Neurodegeneration

To explore the consequences of the neuronal elimination of Dicer in the adult brain bypassing the early embryonic lethality observed in conventional KO mice, we generated inducible forebrain-restricted *Dicer1* mice (referred to as Dicer-ifKO mice) by crossing the CaMKIIa-creERT2 (Erdmann et al. 2007) and *Dicer1*$^{f/f}$ (Harfe et al. 2005) strains. In these mice, the elimination of exon 24, which encodes part of the second RNase III domain, can be induced at any time in recombinase-expressing neurons by tamoxifen administration (Harfe et al. 2005). This recombination is predicted to cause the production of a truncated Dicer protein that lacks RNase III activity (Fig. 1*A*). The recombination of the *Dicer1* locus was confirmed in genomic DNA from the hippocampus and cortex of mutant mice (Fig. 1*B*), the 2 main brain areas expressing the Cre recombinase (Fig. 1*C*). In contrast, we did not observe recombination in the cerebellum in which the CaMKIIα promoter is not active (Fig. 1*B*). Furthermore, exon-specific RT-qPCR assays demonstrated the production of the truncated transcripts in regions showing recombination (Fig. 1*D*). As expected, the reduction of the wild-type allele was only around 50% because Dicer is selectively eliminated in principal neurons, whereas glial cells, interneurons, and other neural types maintain normal expression of the gene. Our analysis of cDNA sequences downstream of the recombination site indicated that the recombined transcripts are stable (Fig. 1*D*, right bar graph). Consistent with this observation and with the production of a C-terminally truncated protein, immunohistochemistry (IHC) experiments only revealed a reduction of immunoreactivity when an antibody raised against the C-terminal domain of Dicer was used (compare Fig. 1*E* with Supplementary Fig. 1*A*). This loss of immunoreactivity was particularly prominent in the dentate gyrus and CA1 subfields (Fig. 1*E* and Supplementary Fig. 1*B*).

Nissl staining analysis of brain anatomy at different time points after tamoxifen treatment revealed that Dicer-ifKO and control littermates were indistinguishable during the first weeks after gene ablation (Fig. 1*F* and Supplementary Fig. 1*C*). However, 12 weeks after treatment, the loss of neurons in the cortex and hippocampus of Dicer-ifKO mice begins to be noticeable. The neurodegenerative process progressed slowly during the subsequent months until the almost complete disappearance of the hippocampal layers 24 weeks after recombination, with the CA3 and CA1 subfields being more affected than the dentate gyrus (Fig. 1*F* and Supplementary Fig. 1*C*,*D*). The thickness of cortical layers was also significantly reduced (Supplementary Fig. 1*C*,*D*).

RT-qPCR assays for the neuropathology markers glial fibrillary acidic protein (GFAP) and major histocompatibility complex I indicated this neuronal loss was associated with neuroinflammation and excitotoxicity (Valor et al. 2010) (Supplementary Fig. 1*E*,*F*). Furthermore, immunostaining against the glial marker GFAP confirmed the neurodegeneration and increased gliosis at late time points after recombination (Fig. 1*G*). Neurodegeneration, however, did not appreciably impact on the animal's survival because more than 80% of mice of both genotypes reached 24 months of age (13 of 16 for controls and 11 of 13 for Dicer-ifKO at 20 months after recombination). In subsequent experiments, we will focus on the early effects of Dicer loss in hippocampal physiology and gene expression prior to neuronal damage.

Dicer Loss Has a Broad Impact on miRNA Biogenesis

To gain additional insight into the consequences of Dicer loss and impaired miRNA biogenesis in mature neurons, we next examined the impact of *Dicer1* ablation on the levels of small RNAs (between 18 and 100 bp) in the hippocampus using next-generation sequencing (NGS). Our analysis revealed that most of the mature miRNAs currently included in miR-Base (1251 of 2035, or 1062 of 1900 after eliminating gene duplications) are expressed in hippocampal tissue (Fig. 2*A*).

As expected, the screen revealed a specific impact on miRNA biogenesis, whereas all other species of small noncoding RNAs were spared (Fig. 2*B*,*C*). Note that by selecting transcripts smaller

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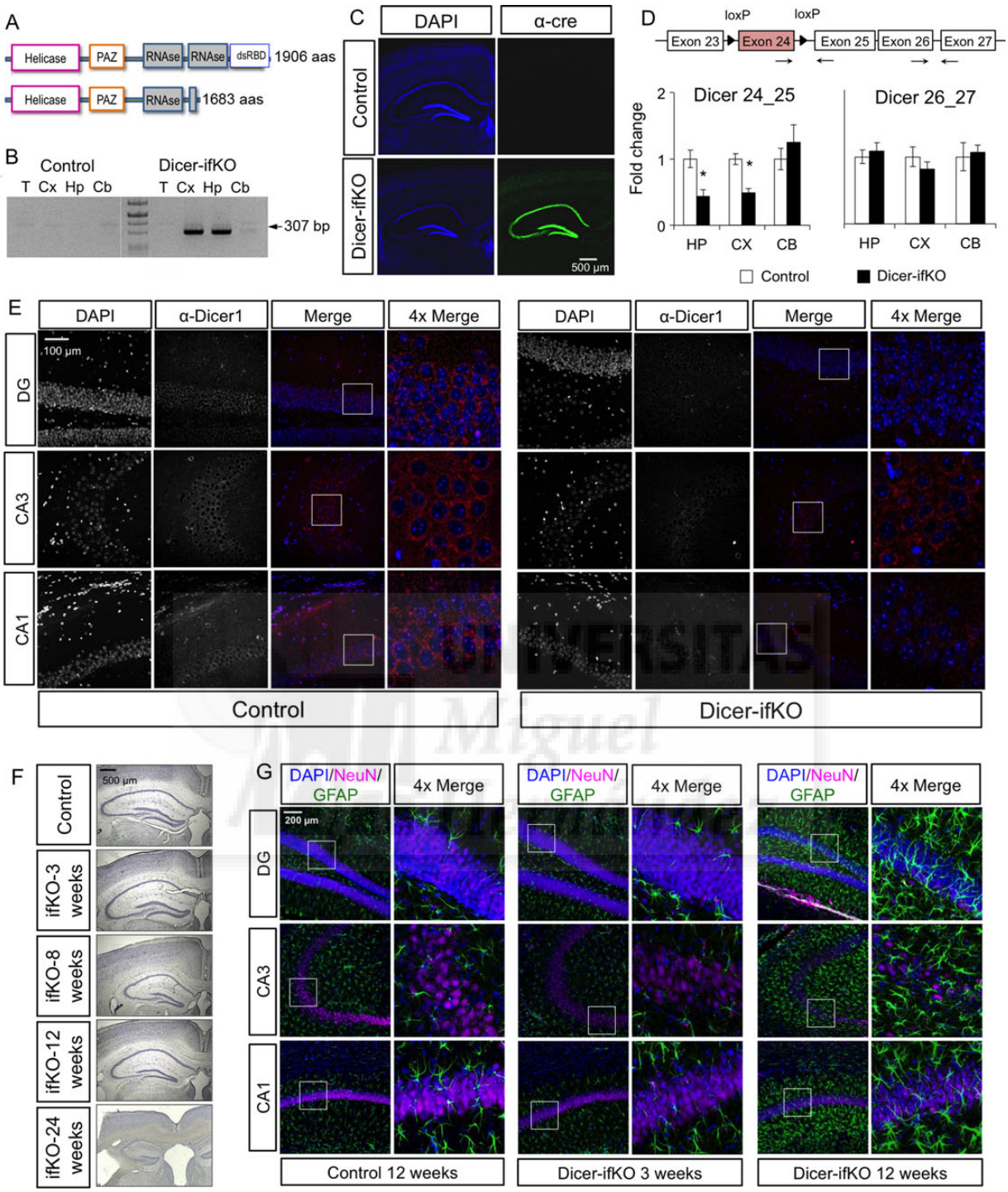

Figure 1. Conditional and inducible *Dicer1* ablation in forebrain neurons. (*A*) Scheme of wild-type Dicer (upper part) and the truncated protein produced after Cre-mediated recombination (lower part). (*B*) PCR analysis on genomic DNA extracted from cortex (Cx), hippocampus (Hp), cerebellum (Cb), and tail (T) of Dicer-ifKO and control littermates revealed the amplification of a recombination-specific band (arrow: 307 bp) only in Cre recombinase-expressing tissues. The predicted amplicon in control animals (1200 bp) was not efficiently amplified under these PCR conditions. (*C*) Representative images of fluorescent immunostaining for Cre recombinase in the hippocampus and cortex of Dicer-ifKO and control littermates. (*D*) RT-qPCR analysis on Dicer mRNAs extracted from the hippocampus (Hp), cortex (Cx), and cerebellum (Cb) of Dicer-ifKO and control littermates using 2 different primer pairs. The upper scheme shows the location of the 2 primer pairs. The pair 24_25 is targeted to sequences in exons 24 and 25 and can only detect wild-type Dicer transcripts, whereas the pair 26_27 is targeted to exons 26 and 27 and detects a sequence present in both normal and truncated transcripts. Control, $n = 6$; Dicer-ifKO mice, $n = 5$; *Significant difference between genotypes evaluated by *t*-test, $P < 0.05$. (*E*) Representative images of immunostaining of brain sagittal sections from control and Dicer-ifKO mice using an antibody that recognizes the C-terminus of Dicer. (*F*) Representative images of Nissl staining in the hippocampus of control and Dicer-ifKO mice at different time points after tamoxifen treatment. (*G*) α-GFAP immunostaining in the hippocampus of Dicer-ifKO and control littermates. The number of glial cells is significantly increased in the hippocampus of Dicer-ifKO mice 12 weeks after tamoxifen administration. Control, $n = 3$; Dicer-ifKO mice, $n = 3$ per time point.

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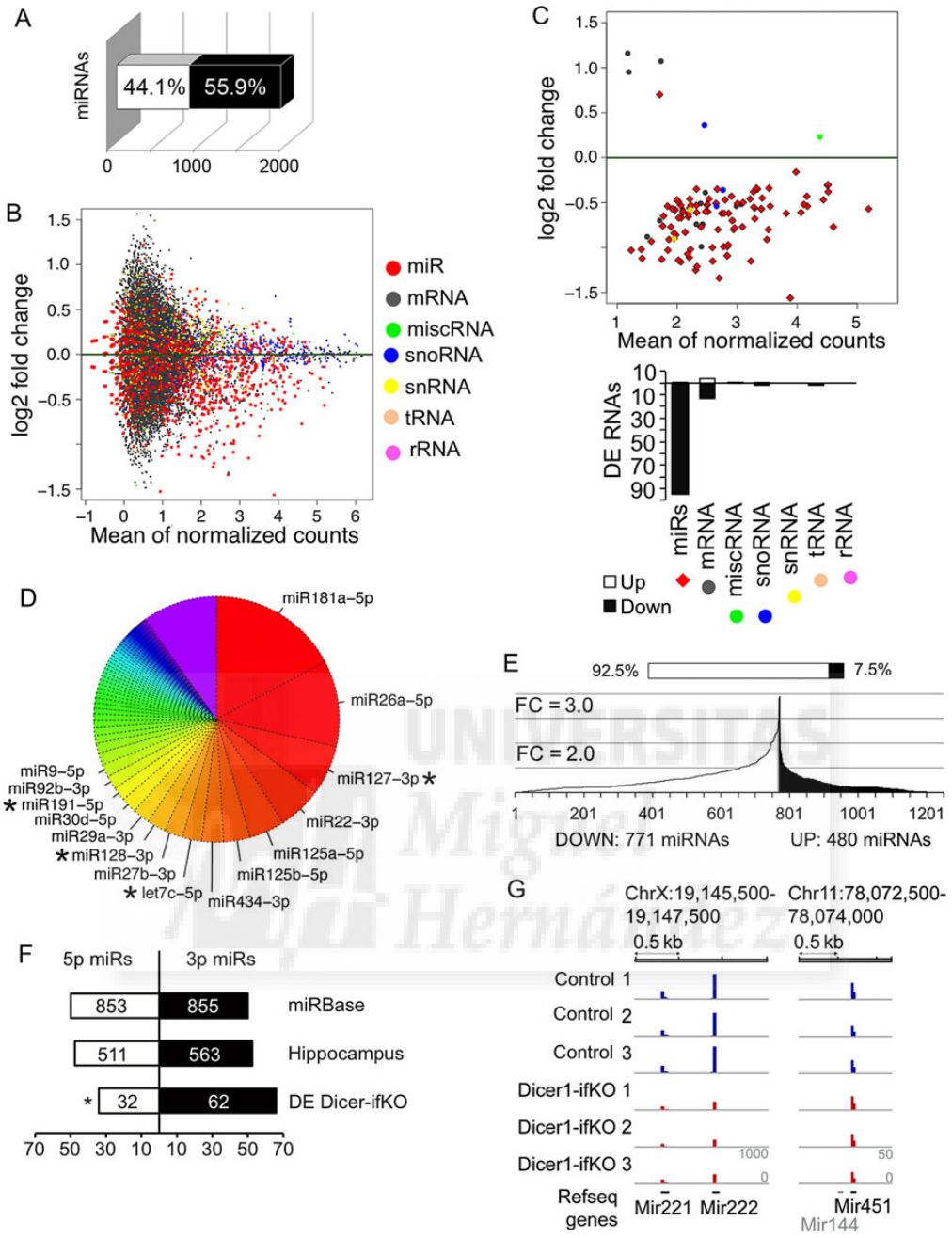

Figure 2. Small RNA-seq screen. (*A*) Percentage of miRNA species included in miR-Base that were detected in hippocampal tissue. The black area in the histogram represents the 1062 species detected in the libraries of control mice. (*B*) Scatter plot representing the normalized counts (expression levels) and fold change values (log_2) in the comparison of RNA-seq profiles from Dicer-ifKO and control mice. Point color defines the different RNA populations analyzed in the experiment. (*C*) Same as in *B* but only significantly affected entities (adj. $P < 0.1$) are shown. The lower bar graph represents the number of differentially expressed (DE) RNAs belonging to each RNA subgroup. (*D*) Pie chart showing the relative abundance of different miRNA species in the mouse hippocampus. The chart shows individual sectors for the 55 most abundant miRNAs, which account for 90% of the reads in the libraries of control mice. The remaining 1007 entities are contained in the purple sector. Asterisks label highly expressed miRNAs that are significantly decreased in Dicer-ifKO mice. (*E*). Distribution of miRNA changes 3 weeks after *Dicer1* ablation. All miRNAs expressed in the hippocampus are separately ordered for upregulation and downregulation based on the absolute fold change value, and each miRNA is plotted as a separate entity on the x-axis with the corresponding fold change represented on the y-axis. The horizontal bar graph shows the relative weight (percentage of the area under the curve) of upregulation and downregulation with FC >2.0. The total number of miRNAs in each group is also indicated. (*F*) Bar graph showing the percentage of 5p and 3p miRNAs annotated in miR-Base (miRBase), those detected in our analysis (Hippocampus) and those found to be differentially expressed in Dicer-ifKO mice (DE Dicer-ifKO). Absolute numbers are also shown inside of the bars. (*G*) Profiles from small RNA-seq screen quantification for the miR-221/miR-222 and miR-144/miR-451 regions.

than 100 bp, we excluded many precursor miRNAs (pre-miRNAs) from our screen. The overall distribution of reads across miRNAs was similar in both genotypes. In both cases, 55 miRNA species accounted for >90% of the total read number in our libraries (Fig. 2*D*). However, there was a strong overall trend toward lower miRNA levels in Dicer mutants (Fig. 2*E*). Approximately 10% of the expressed miRNAs were significantly reduced (adj. $P < 0.1$) in Dicer-ifKO mice 3 weeks after triggering gene ablation

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(Fig. 2C and Supplementary Table 1). Apart from the rare instances of Dicer-independent maturation (Ha and Kim 2014), such as miR-320 and miR-451 (which are not affected in Dicer-ifKO mice), the fact that a percentage of miRNAs were not apparently reduced could be explained by their reported high stability (Gantier et al. 2011), the cell specificity of the deletion and the presence of residual amounts of Dicer in Dicer-ifKO mice at this early time after recombination (Fig. 1E). Those miRNAs that were significantly downregulated (95 species) may be particularly sensitive to Dicer loss because of their faster turnover. Downregulation affected both 5p and 3p species, although we observed a significant bias toward 3p miRNAs (Fig. 2F: $P < 0.01$, Fisher's exact test). Overall, our screen clearly illustrates a range of expression and stability between miRNAs.

The list of affected miRNAs included both recently identified miRNAs, such as miR-6944, and previously investigated miRNAs that are known to play relevant functions in neurons. For example, the miRNA that presented the largest and most significant change in expression was miR-222 that modulates PTEN-Akt signaling and neurite outgrowth (Zhou et al. 2012) (Fig. 2G, 3-fold downregulation). Other important affected miRNAs were miR-138 and miR-134 that regulate spinogenesis (Schratt et al. 2006; Siegel et al. 2009), miR-124 that constrains synaptic plasticity in *Aplysia* neurons (Rajasethupathy et al. 2009), miR-128 that contributes to the regulation of neuronal excitability (Tan et al. 2013), and the activity-regulated miR-212 that has been involved in several forms of neuroplasticity (Wanet et al. 2012).

Early Dicer Loss Affects Neuronal Plasticity and Excitability-Related Genes

We conducted a second RNA-seq-based screen for larger RNA molecules bearing a polyA tail to examine the impact of diminished miRNA production on mRNAs. Our differential screen, conducted early after gene ablation, overcomes the confounding effect introduced by changes in the cellular composition of the tissue and therefore is likely to retrieve direct miRNA targets. According to our filter (adj. $P < 0.1$), approximately 300 protein-encoding genes were differentially expressed in the hippocampus of Dicer-deficient mice 3 weeks after recombination. Both upregulation and downregulation of genes were observed. However, consistent with the notion that miRNAs are constrainers for the expression of target genes, there was a significant bias toward upregulation, especially when only the largest changes (FC >1.2) were considered (Fig. 3A,B and Supplementary Table 2). We should, however, note that our screen only revealed very modest changes in transcript levels that rarely surpassed 20%. Because miRNAs are thought to affect both the stability and the translational rate of target mRNAs, it is possible that the modest changes in transcript levels led to larger variations at the protein level.

The largest changes were directly related to the genetic manipulation used to generate Dicer-ifKO mice. Thus, the 3 genes contained in the BAC bearing the CaMKIIa-creERT2 transgene (*Arsi*, *CamK2a*, and *Slc6a7*) were upregulated in Dicer-ifKO mice. Additionally, the exon of the *Esr1* gene encoding the estrogen-binding domain fused to Cre recombinase to produce the chimeric creERT2 showed a dramatic signal increase, reflecting transgene expression (Fig. 3C and Supplementary Fig. 2A). We also detected a significant increase in Dicer1 transcripts skipping exon 24 (as expected after recombination) or bearing the intronic sequence located between exons 23 and 24 (likely as a consequence of the loss of the acceptor site located in exon 24) (Fig. 3D).

In addition, we detected a number of changes that suggest a compensatory response to Dicer loss. Thus, among the genes upregulated in Dicer-ifKO mice, we find several genes encoding key components of the RNA-induced silencing complex (RISC) that are downstream of Dicer, such as Eif2c1 (*aka Ago1*, encoding Argonaute 1, which is one of the proteins directly responsible for silencing target mRNAs), its binding partner Tnrc6c and the Cnot1 subunit of the CCR4–NOT complex implicated in miR-mediated translational repression (Fabian et al. 2011). The gene *Adarb1* that encodes an adenosine deaminase homologous of ADAR1, which interacts with Dicer to facilitate RISC loading and RNA silencing (Ota et al. 2013), was also upregulated. RT–qPCR assays using independent samples confirmed the upregulation of components of the miRNA biogenesis pathway (Fig. 3E). This raises the possibility that the absence of Dicer activates or potentiates alternative pathways for miRNA processing (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang et al. 2010).

Other detected changes, especially among upregulated genes, are likely to be the direct consequence of the decrease in inhibitory miRNAs. Notably, the WebGestalt (Wang et al. 2013) analysis of the enrichment of miRNA targets among the genes that were upregulated in Dicer-ifKO mice identified more than 70 miRNAs or miRNA families exhibiting highly significant enrichments (adj. $P < 10^{-5}$). However, the same threshold did not retrieve any significant association for downregulated genes (Fig. 3F and Supplementary Table 3). The overall changes associated with these miRNAs and miRNA families covered more than 70% of the upregulated genes detected in our screen. Furthermore, we observed a large overlap (>50%) between the predicted affected miRNAs according to WebGestalt and the differentially expressed miRNAs identified in our small RNA-seq screen. These 2 results strongly support the identification of direct miRNA targets. Interestingly, a number of genes that were upregulated in Dicer-ifKO mice, such as the aforementioned *Eif2c1*, the intellectual disability-associated genes *Srgap3* and *Slc6a8*, and those encoding for the kinase Hipk1, the RNA helicase Ddx3x and the neuronal cell surface protein neurexin 3, are the targets of multiple miRNAs that were significantly downregulated in these mice.

Further functional analyses using WebGestalt and DAVID (Huang et al. 2009) highlighted the particular relevance for neuronal function and behavior of the genes that were differentially expressed in Dicer-ifKO mice (Supplementary Table 4). Intriguingly, the list of affected "Molecular functions" was dominated by terms related to "Purine ribonucleotide/ribonucleoside metabolism" (*P*-values ranging up to 10^{-9}), which were associated with more than 20% of differentially expressed genes. This prominence was observed in both downregulated and upregulated genes ($P \sim 10^{-5}$). The enrichment analysis also retrieved biological processes that were differentially affected in either one of these gene subsets. Thus, in line with the regulatory role of miRNAs in translation, the Gene Ontology (GO) terms "Translation" and "Ribosome" were selectively enriched among downregulated genes. In contrast, "neurotrophin signaling pathway," "synapse," and numerous "phosphorylation" and "protein kinase"-related terms were selectively enriched among upregulated genes, which suggest that neuronal responsiveness may be altered as a result of interfering with miRNA production. Consistent with this view, the enrichment for phenotype-related genes revealed significant associations with "Abnormal synaptic transmission" (adj. $P = 5 \times 10^{-7}$), "Abnormal synaptic plasticity" (adj. $P = 2 \times 10^{-4}$), and "Seizures" (adj. $P = 3 \times 10^{-4}$). Notably, more than a dozen genes related to seizures were differentially expressed in the hippocampus of Dicer-ifKO mice, including *Cacna1g*, *Akt3*,

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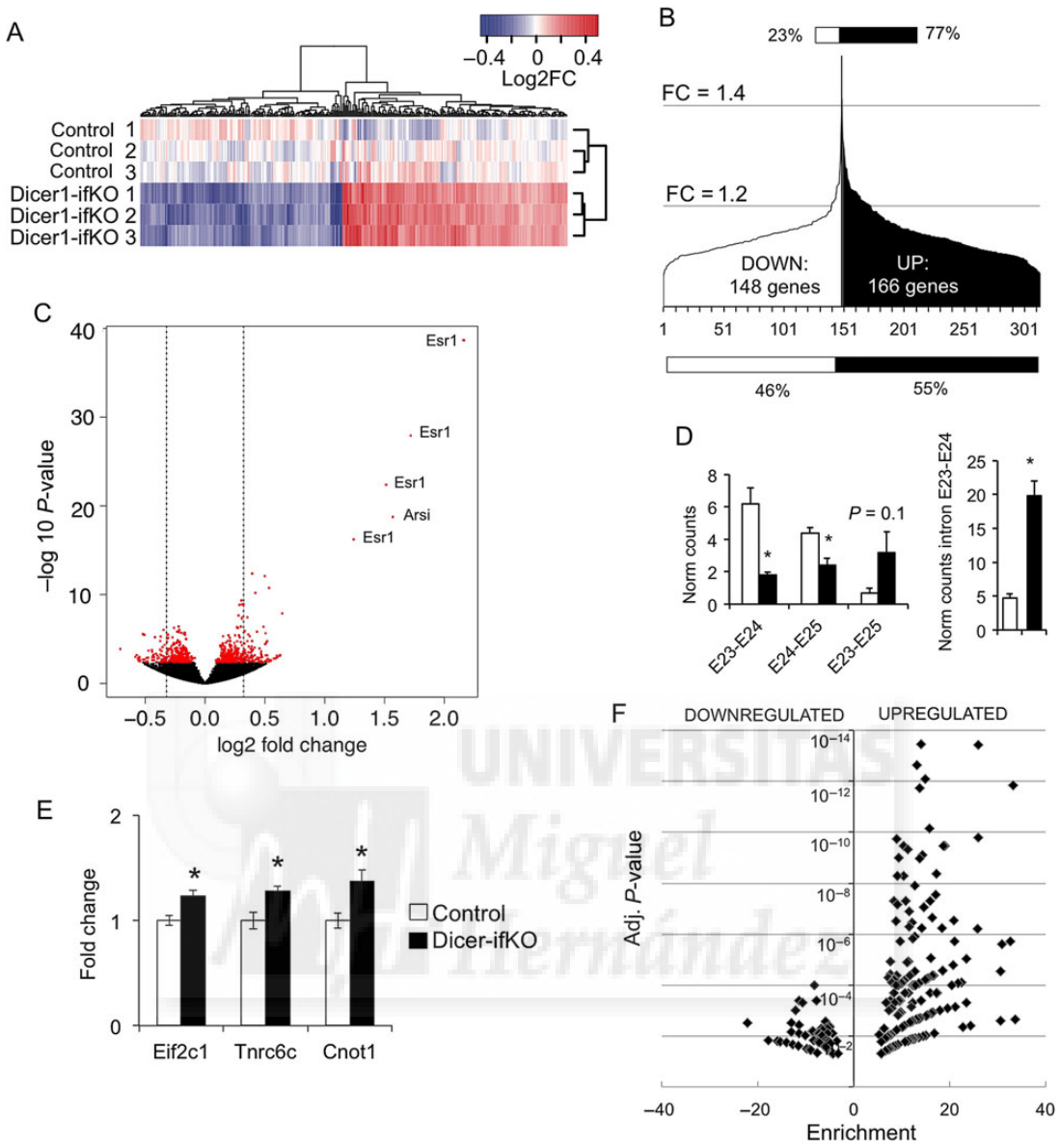

Figure 3. mRNA-seq screen. (*A*) Hierarchical clustering of the level of change for differentially expressed mRNAs (adj. $P < 0.1$) in Dicer-ifKO mice. (*B*) Distribution of mRNA changes 3 weeks after *Dicer1* ablation. Genes are separately ordered for upregulation and downregulation based on their absolute fold change, and each gene is plotted as a separate entity on the *x*-axis with the corresponding fold change represented on the *y*-axis. The 2 horizontal bar graphs refer to the relative weight of upregulation and downregulation considering all of the genes (lower graph) or only those with FC >1.2 (upper graph). The total number of genes affected is also indicated. (*C*) Volcano plot of fold change values ($\log_2$) and raw *P* values ($-\log_{10}$) of differentially regulated transcripts in Dicer-ifKO mice (red dots: adj. $P < 0.1$). (*D*) Normalized counts for specific exon connections and intronic sequences that were significantly altered in Dicer-ifKO mice. (*E*) RT-qPCR for components of the RISC complex. $^*P < 0.05$ (*t*-test between genotypes); $n = 4$ for both genotypes. (*F*) miRNA–target enrichment analysis for the downregulated and upregulated gene subsets. The volcano plot represents the fold enrichment and associated adj. *P*-value for the different miRNAs and miRNA families that were significantly enriched for targets (adj. $P < 0.05$) using *WebGestalt*.

Kcnt1, *Hcn2*, and *Bsn* (Supplementary Fig. 2*B*). For instance, the elevation of the $\alpha 1_G$-mediated low-voltage-activated calcium current (encoded by *Cacna1g*) has been shown to increase the excitability of CA1 pyramidal neurons (Kratzer et al. 2013) and causes absence epilepsy in mice (Ernst et al. 2009), Akt3 is a serine-threonine protein kinase involved in the control of synaptic strength (Wang et al. 2003) and epilepsy (Tokuda et al. 2011), and Kcnt1 is a sodium-gated potassium channel important for K^+-hyperpolarizing current that has been recently implicated in seizure and epilepsy pathogenesis (Barcia et al. 2012; Kingwell 2012).

Dicer-ifKO Mice Exhibit an Exacerbated Response to Kainic Acid and Novelty

To investigate whether seizures and neuronal responsiveness were indeed enhanced after Dicer loss, we next compared the response of mutant and control littermates to kainic acid (KA). This analog of L-glutamate causes massive neuronal depolarization and, consequently, severe epileptic seizures (Ben-Ari 1985). We found that Dicer-deficient mice were much more sensitive to this compound than their control siblings. The same dose of kainate elicited more severe seizures in mutant mice than in control

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littermates, as determined by forelimbs clonus, rearing and falling and, occasionally, death (Fig. 4A).

KA-evoked seizures initiate a well-established transcriptional response in neurons that includes many IEGs, such as those encoding the transcription factors Fos and Npas4 and the cytoskeleton-associated protein Arc. These genes represent the nuclear response to the activation of signal transduction cascades by synaptic activity and are thought to play important roles in neuronal survival and plasticity (Flavell and Greenberg 2008; Benito and Barco 2014). We examined the induction of the aforementioned IEGs by KA and found that, in agreement with RNA-seq data, their basal expression was similar in both genotypes. In contrast, their expression 2 h after seizure was increased up to 2-fold in Dicer-ifKO mice (Fig. 4B), which is consistent with seizure severity.

Because the increase in IEG induction observed in the KA paradigm could be a consequence of the stronger seizures, we next explored whether activity-driven gene expression in the hippocampus of Dicer-ifKO mice was also enhanced in other paradigms. Toward this end, we investigated the hippocampal induction of 5 IEGs (*Fos*, *Npas4*, *Arc*, *Egr1*, and the activity-regulated miRNA *miR212*) in response to the exploration of a novel environment (NE) (Fig. 4C), a process that is required for the consolidation of hippocampal-dependent memory (Lisman and Grace 2005; Moncada and Viola 2007). RT-qPCR analyses showed a strong IEG induction in both genotypes (Fig. 4D–H). As expected, Dicer-ifKO samples showed significantly higher levels of the precursor form of miR212 (pre-miR212) both in basal and NE conditions as a consequence of impaired Dicer-mediated maturation (Fig. 4H). Furthermore, the 5 IEGs were more induced in Dicer-ifKO mice than in control littermates, although for *Fos* this trend was not significant (Fig. 4E). To examine Fos induction in greater detail, we used IHC and found that although novelty-induced Fos expression in numerous CA1 pyramidal neurons and scattered granule cells of the dentate gyrus in both genotypes, a larger number of novelty-induced Fos-positive cells were observed in both regions for Dicer-ifKO mice (Fig. 4I,J). We next examined whether the duration of IEG induction by novelty was altered by Dicer loss by measuring transcript levels 1 h after returning the mice to their home cage (HC). *Fos*, *Npas4*, and *Egr1* returned to basal levels while some induction was still detected for *Arc* and *pre-miR212*. However, no genotype effect was detected at this time point (Fig. 4D–H). These results suggest that the enhanced IEG induction was originated by both an increase in the number of responding neurons, resulting from a reduction in the threshold for activation, and a stronger transcriptional response in activated neurons.

Excitability Is Increased in Neurons Lacking Dicer

To directly examine whether neuronal excitability was altered as a result of impaired miRNA production, we conducted intracellular recordings in hippocampal CA1 neurons of Dicer-ifKO and control littermates 3 weeks after gene ablation. We did not observe any significant difference in the I/O curve (Fig. 5A) nor in the resting potential and resistance of CA1 neurons (Fig. 5B). However, the number of action potentials elicited by a depolarizing current injection (100 pA, 500 ms) was significantly higher in Dicer-ifKO mice (Fig. 5C,D), confirming an increase in the excitability of CA1 pyramidal neurons from Dicer-ifKO mice. Notably, the increase in the firing frequency during the first action potential (Fig. 5E) was associated with reduced medium after-hyperpolarization (mAHP) (Fig. 5F,G). The increase in the excitability of CA1 pyramidal neurons and the concomitant decrease in mAHP could be caused by the altered expression of some of the excitability and seizure-related genes retrieved in our mRNA-seq screen, such as *Cacna1g* and *Akt3*. RT–qPCR assays confirmed the upregulation of these genes (Fig. 5H). These results demonstrate that neuronal excitability is enhanced upon Dicer ablation and provide an appropriate framework for the interpretation of the diverse phenotypes observed in Dicer-ifKO mice, including the enhanced response to KA and novelty and the late excitotoxicity-related neurodegeneration.

Dicer-ifKO Mice Show a Transient Enhancement of Fear Memory

In retrospect, our finding could also explain the enhanced memory phenotype reported by Konopka et al. (2010) in a closely related mouse strain. We next examined if memory formation was similarly enhanced in Dicer-ifKO mice. Toward this end, we examined their performance in contextual fear-conditioning (FC), a memory task that measures the ability of the animals to associate an aversive stimulus (mild foot shock) with neutral environmental cues (Fanselow and Poulos 2005). Our experiment revealed that Dicer-ifKO mice exhibit a stronger memory for the shock, manifested as increased freezing behavior (Fig. 6A,B). Importantly, the initial response of the mice to the shock was similar in both genotypes, suggesting that the enhanced freezing was not caused by enhanced nociception. At this time point, mutant and control mice also exhibited similar behaviors in the open field (OF) and in the elevated plus maze (EPM) tasks, 2 behavioral tests that evaluate locomotor activity, exploratory behavior, and anxiety (Fig. 6C–E). This excludes the possibility that the differences in FC were caused by differences in mobility or anxiety levels.

We next investigated whether the memory enhancement observed in Dicer-deficient mice was not only more robust than in control mice but also more resistant to forgetting or extinction, a question not considered in the previous study. Toward this end, we evaluated the same animals 1 and 2 weeks after training. Notably, whereas control animals exhibited a progressive reduction of freezing after repeated exposures to the context in the absence of shock, Dicer-ifKO mice did not show any reduction (Fig. 6B, $F_{1,16\ \text{genotype}} = 17.11$, $P < 0.001$, $F_{2,32\ \text{time} \times \text{genotype}} = 4.55$, $P = 0.02$).

Dicer-ifKO Mice Show a Rapid Increase in Body Weight After Gene Ablation

In the course of these experiments, we noticed a striking novel phenotype of Dicer-deficient mice. Dicer-ifKO mice almost doubled their weight in the few weeks immediately following gene ablation (Fig. 7A). Notably, the rapid increase in body weight was followed by a slower and progressive weight loss that returned the body weight to control values 9 weeks after triggering recombination (Fig. 7B).

We next examined if the Cre recombinase transgene used in our study was expressed in some of the hypothalamic nuclei that regulate feeding behavior and detected its expression in the arcuate nucleus (ARC), a structure of the mediobasal hypothalamus that plays a critical role in regulating food intake (Fig. 7C and Supplementary Fig. 3A). This nucleus contains neurons positive for the orexigenic peptides NPY and Agrp that induce ravenous eating when activated (Aponte et al. 2011). We therefore hypothesized that the weight gain phenotype of the Dicer-ifKO mice could be caused by an increase in the activity of ARC orexigenic neurons triggered by Dicer loss. Importantly,

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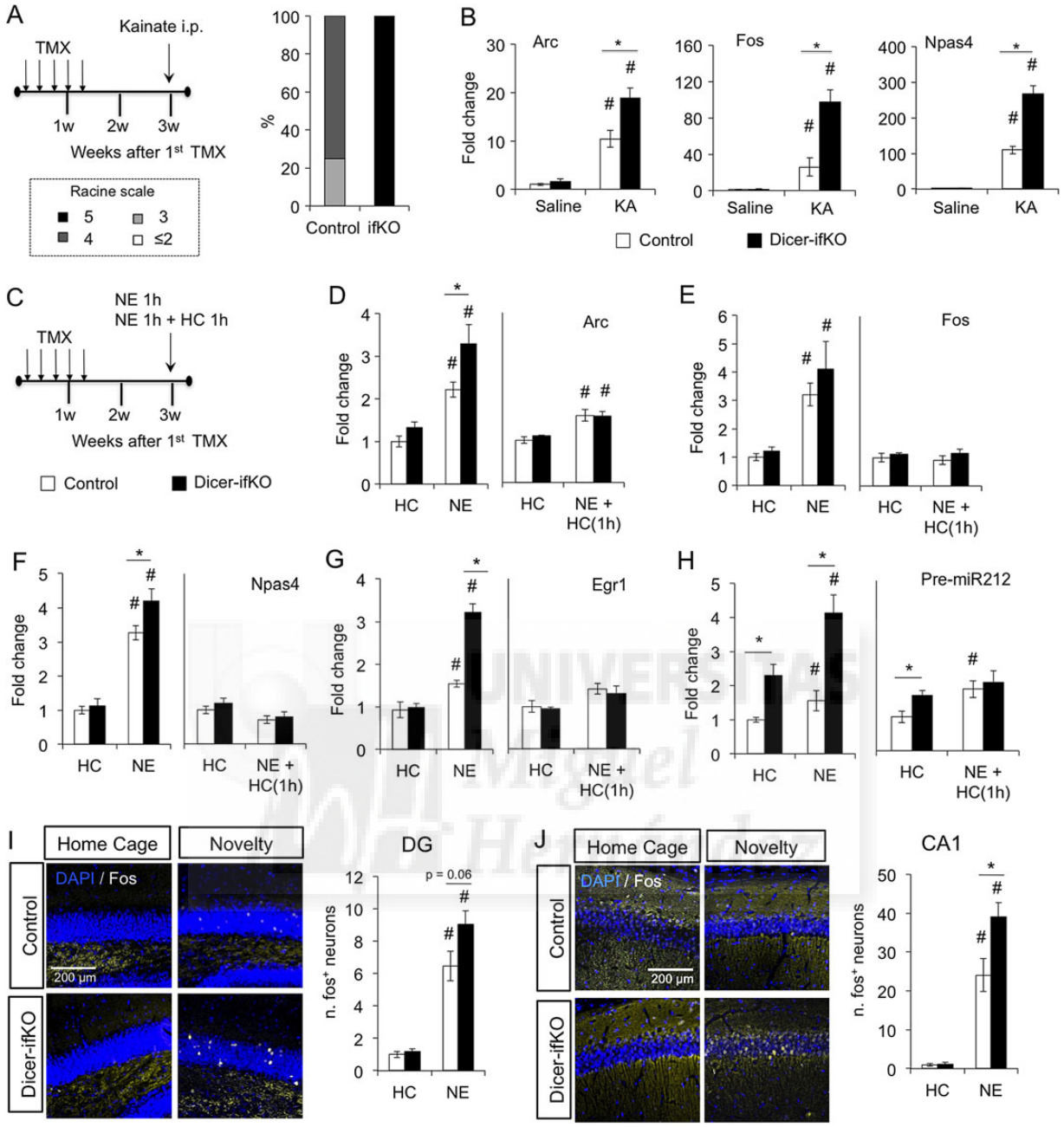

Figure 4. The loss of Dicer in principal neurons enhanced kainate-induced seizures and IEG induction. (*A*) Left scheme: Experimental design. Right graph: Responses to KA (15 mg/kg) 2 h after i.p. injection were evaluated using the Racine scale. (*B*) Kainate-induced IEG mRNA levels in controls and Dicer-ifKO mice 3 weeks after tamoxifen. Control and Dicer-ifKO mice were injected with saline (control, $n = 7$ and Dicer-ifKO, $n = 6$) or KA (control, $n = 5$ and Dicer-ifKO, $n = 9$). $^{*}P < 0.05$ in *t*-test between genotypes; $^{\#}P < 0.05$ in *t*-test between treatments. (*C*) Experimental design of the novelty exposure experiment. (*D–H*) Novelty exposure (NE) for 1 h induces the expression of the IEGs *Arc* (*D*), *Fos* (*E*), *Npas4* (F), *Egr1* (*G*), and *miR212* (*H*). Dicer-ifKO mice consistently show stronger IEG induction after 1 h of NE, but no difference between genotypes was observed after an additional hour in the home cage (HC). Note that pre-miRNA levels of activity-induced miR-212 are significantly different in Dicer-ifKO mice. The numbers of mice used in the NE (1 h) experiment were as follows: control ($n = 7$) and Dicer-ifKO mice ($n = 7–8$). The numbers of mice used in the NE (1 h)+HC (1 h) experiment were as follows: control ($n = 4–6$) and Dicer-ifKO ($n = 4–5$). (*I–J*) Fos-positive neurons were significantly increased in the DG (*I*) and CA1 (*J*) of Dicer-ifKO mice after NE. Control $n = 4$ and Dicer-ifKO $n = 4$ in both conditions (4 slices per mouse were used in the quantification). $^{*}P < 0.05$ (*t*-test between genotypes); $^{\#}P < 0.05$ (*t*-test between treatments).

we observed increased gliosis at the ARC and enlargement of the third ventricle at later time points indicating that this structure may also undergo neurodegeneration (Supplementary Fig. 3B,C).

To confirm whether the orexigenic response was increased in Dicer-deficient mice, we examined food intake both in basal and stimulated conditions. The measurement of daily food consumption at different time points after recombination strongly correlated with weight gain (Fig. 7D). In contrast, feeding behavior in response to 15 h of food deprivation was similar in control and Dicer-ifKO mice (Fig. 7E). These results indicate that orexigenic activity regulating the homeostatic hunger-satiety cycle is altered in mice lacking Dicer, but a potent orexigenic stimulus, such as 15 h of starvation, can overcome this difference. Notably, 4 weeks after gene ablation, coinciding with the strongest weight

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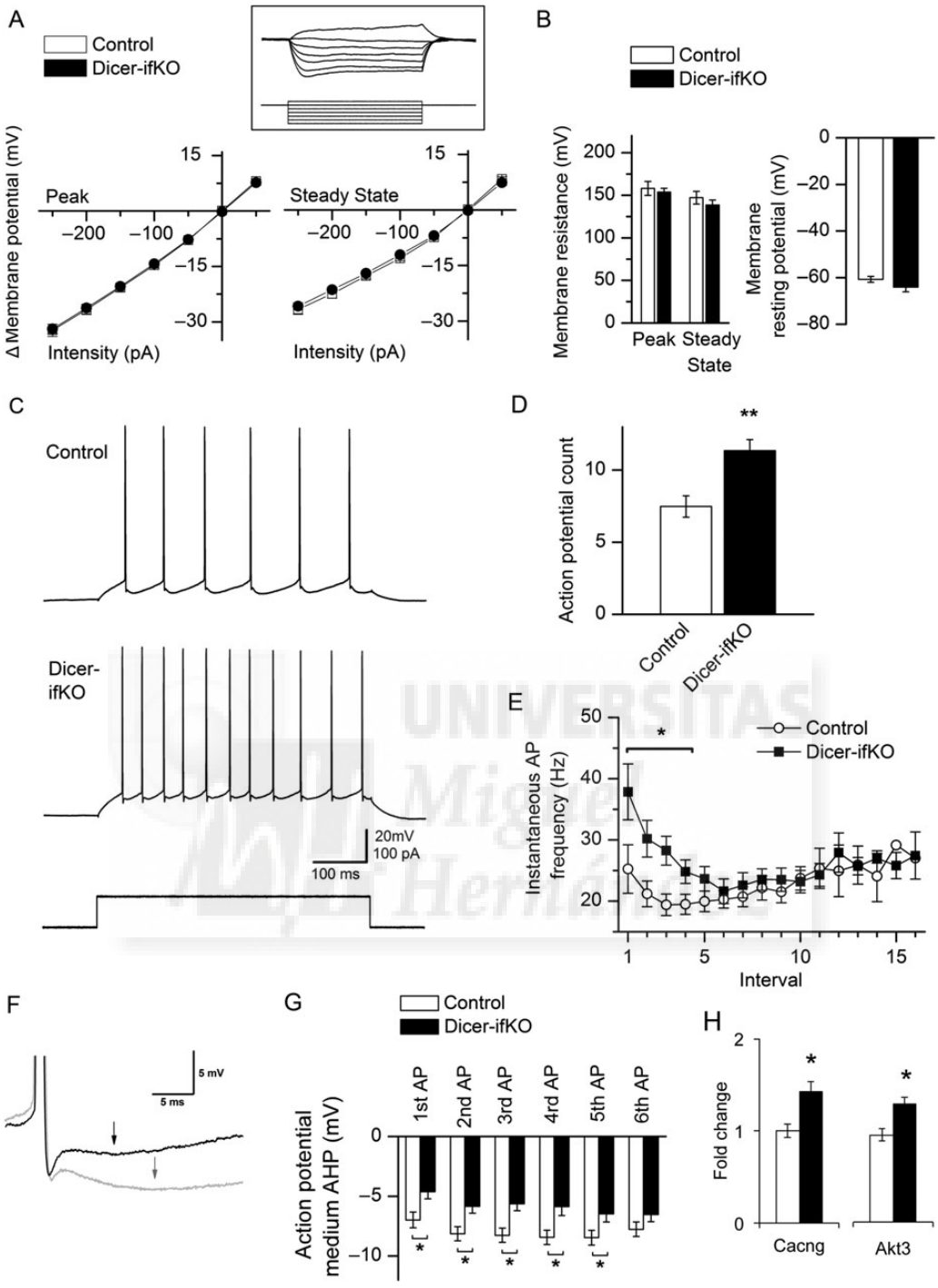

Figure 5. Increased excitability of CA1 pyramidal neurons in Dicer-ifKO mice. (*A*) Current–voltage relationship curves showed no differences between control and Dicer-ifKO mice. The upper inset shows an example of the neuronal response (top) to the pulse protocol (bottom). (*B*) Membrane resistance and membrane resting potential were not different between groups. (*C*) Examples of responses to a 100-pA current depolarizing pulse (bottom) in CA1 pyramidal neurons from control and Dicer-ifKO mice. (*D*) Average number of action potentials fired during the 500 ms duration of the 100-pA current pulses ($P = 0.001$, $n = 29$ control, 17 mutant). (*E*) Average of the instantaneous frequency, calculated between each pair of action potentials (intervals), in response to depolarizing current pulses ($n = 19$–28). (*F*) Detail of the first action potentials fired in the examples of (*C*). The arrows mark the point where after-hyperpolarization measurements were made (20–50 ms after each action potential). The gray trace corresponds to a control mouse and the black trace to a Dicer-ifKO mouse. (*G*) Average of the medium after-hyperpolarization current on each of the first 6 action potentials in Dicer-ifKO and control mice ($n = 27$ control, 17 Dicer-ifKO). (*H*) RT-qPCR for 2 relevant excitability-related genes ($n = 4$ for both genotypes). *$P < 0.05$ in *t*-test between genotypes.

gain, the values for basal and starvation-induced food intake were similar. This result suggests a chronic activation of the ARC nucleus. In agreement with this view, the injection of ghrelin, which mimics a physiological orexigenic stimulus (Morton et al. 2014), induced food intake in both mutant and control animals, but the values for basal and ghrelin-induced food intake were similar in Dicer-ifKO mice (Fig. 7*F*). Interestingly, our transcriptomics screen, although conducted in the hippocampus,

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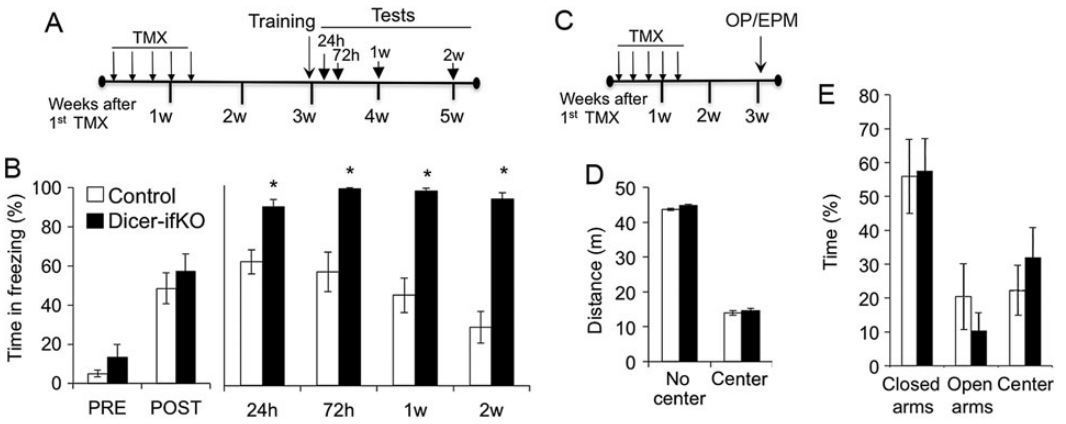

Figure 6. Dicer loss enhanced fear memory. (*A*) Scheme presenting the different phases of the fear-conditioning experiment. (*B*) Dicer-ifKO mice showed more freezing in all test sessions in a fear-conditioning experiment. Control: $n = 9$; Dicer-ifKO mice: $n = 10$. (*C*) Experimental design for the open-field (OF) and elevated plus maze (EPM) experiment. Different cohorts of mice were evaluated in these 2 tasks. (*D*) No difference between control and Dicer-ifKO mice was observed in the OF test. Control: $n = 16$; Dicer-ifKO: $n = 13$. (*E*) No differences between genotypes were observed either in the EPM test. Control: $n = 10$; Dicer-ifKO: $n = 10$. In all graphs, $^{*}P < 0.05$ (t-test between genotypes).

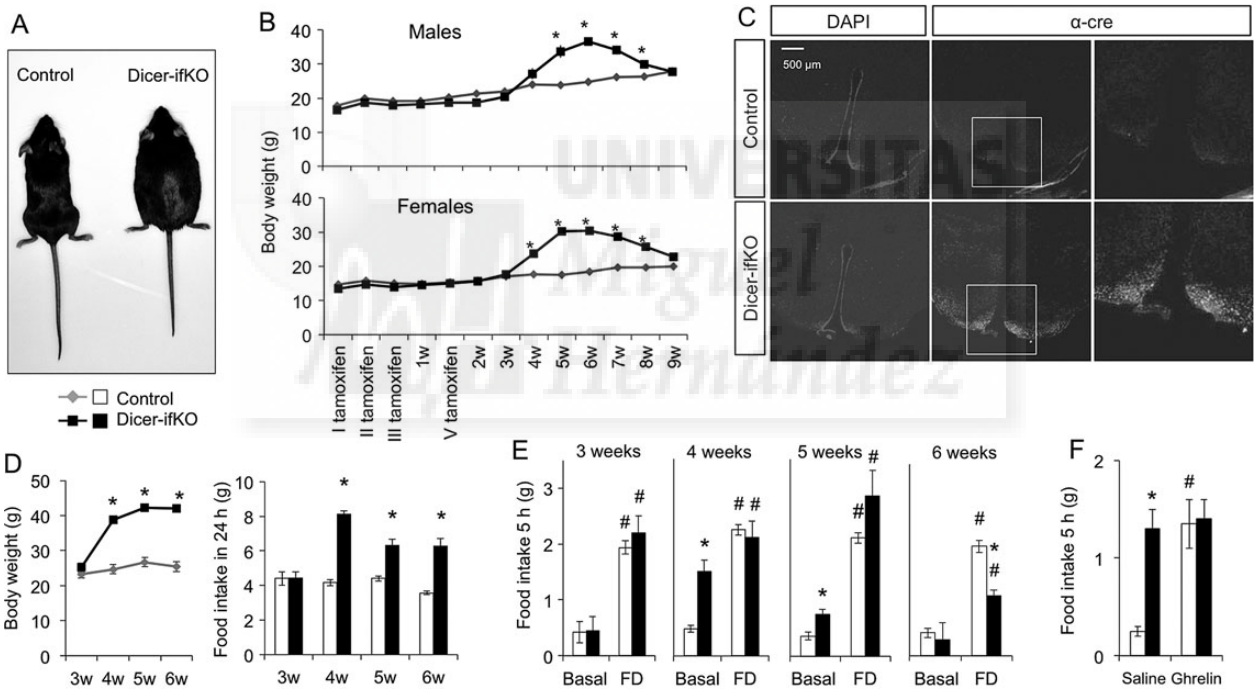

Figure 7. Dicer loss caused rapid changes in body weight. (*A*) Representative image of a Dicer-ifKO (left) and a wild-type mouse (right) 8 weeks after recombination. (*B*) Both male (upper panel) and female (lower panel) Dicer-ifKO mice showed a dramatic and transient increase in body weight. (*C*) Immunostaining for Cre recombinase in of the arcuate nucleus of Dicer-ifKO and control mice 3 weeks after tamoxifen administration. See Supplementary Figure 3A for additional detail. (*D*) Weight variations in Dicer-ifKO mice correlated with altered food intake. (*E*) Food deprivation (FD)-induced feeding was not altered in Dicer-ifKO mice. (*F*) Ghrelin-induced feeding was not affected by Dicer loss. Control: $n = 4$–5; Dicer-ifKO: $n = 5$; $^{*}P < 0.05$ (t-test between genotypes); $^{\#}P < 0.05$ (t-test between treatments).

retrieved a number of upregulated genes in the mutant mice that are related to the regulation of food intake, such as an NPY receptor and several proteins in the PTEN-Akt signal transduction cascade (Vinnikov et al. 2014).

Discussion

The combination of state-of-the-art genomic screens with a comprehensive phenotypic characterization provided unprecedented insight into the changes in the RNA landscape that result from Dicer loss in mature neurons and their consequences in different aspects of animal's physiology and behavior. Two complementary differential screens for changes in small RNA species and mRNA identified specific molecules and biological processes affected by this genetic manipulation. Although previous experiments in other mouse strains lacking Dicer in forebrain neurons have also reported changes in miRNAs (Babiarz et al. 2011) and their potential targets (Dorval et al. 2012), those screens were not conducted in parallel and did not discriminate against indirect changes related to ongoing neurodegeneration. Our

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experiments, conducted at a time in which neither degeneration nor gliosis were observed, revealed a striking array of phenotypes that are consistent with an increase in neuronal responsiveness.

As outlined in the Introduction section, although miRNAs have been implicated in a broad range of physiological and pathological processes both during embryonic development and in adults (Ameres and Zamore 2013; Gurtan and Sharp 2013; Hausser and Zavolan 2014), their specific roles and modes of action are still under debate. Two nonexclusive views have emerged from the rapid progress in this area of research during the last decade. On the one hand, numerous studies have focused on binary miRNA–target interactions depicting relatively simple molecular cascades that contrast with the large number of potential targets for each miRNA predicted in silico. On the other hand, computational models and systems biology analyses propose that the miRNA system, as a whole, may confer robustness to biological processes by reinforcing and buffering transcriptional programs (Osella et al. 2011; Pelaez and Carthew 2012). Although these 2 models are not mutually exclusive, they lead to different predictions concerning the consequences of interfering with the miRNA system. If serving robustness were the main function of miRNAs, major perturbations of the system in steady-state organisms could be absorbed without overt or immediate consequences in many cellular functions (Inui et al. 2010). In mature neuronal circuits, the buffering role of miRNAs is likely to primarily influence biological processes that rely on acute transcriptional waves, such as those induced in neurons in response to stimuli, and on cycling feedback loops, such as feeding behavior.

Numerous studies have shown the relevance of individual miRNAs in neuronal plasticity and memory (Kosik 2006; Vo et al. 2010; Fiore et al. 2011; Saab and Mansuy 2014). The phenotypes observed in Dicer-ifKO mice may originate from the dysregulation of a single (or a few) miRNA–target pair, as recently proposed in the case of hyperphagia (Vinnikov et al. 2014), or be the result of an overall decrease in miRNA abundance and reduced robustness (according to the buffering role discussed above). Although our experiments do not allow us to conclusively discriminate between these 2 possibilities, it is noteworthy that the phenotypes detected in our comprehensive characterization are consistent with the robustness model. First, we reported several novel hippocampal-related alterations associated with early Dicer loss (namely, enhanced seizure susceptibility, stronger IEGs induction, and increased excitability of CA1 pyramidal neurons) that indicate that neuronal responsiveness to transient incoming signals was boosted as a result of blocking miRNA biogenesis. The increased levels of IEG induction after neuronal stimulation (by kainate or novelty) could reflect the inability of the neurons to adapt their response to fluctuations in gene expression. Other studies have also revealed miRNA-dependent phenotypes that were only manifested upon the application of certain stimuli or stresses (Miska et al. 2007; Li et al. 2009; Herranz and Cohen 2010). Second, and closely related to enhanced responsiveness, we confirmed the paradoxical memory enhancement phenotype associated with early gene ablation (Konopka et al. 2010) and demonstrated that formed memory was not only stronger but also more resistant to extinction. Third, as in previous studies in other conditional KO strains (Davis et al. 2008; Hebert et al. 2010; Konopka et al. 2010), our experiments revealed a requirement for Dicer in long-term survival of forebrain principal neurons. We show that the slow neurodegeneration of sensitive structures, such as the hippocampus, was associated with gliosis and the expression of excitotoxicity markers (Valor et al. 2010), which again connects to aberrant responsiveness. Together, our results suggest that the Dicer/miRNA system may play a critical role in setting a threshold for neuronal activation in the hippocampus that has 2 sides: it constitutes a molecular brake for memory processes and protects hippocampal neurons against overactivation.

In parallel to the changes in the hippocampus, Dicer-ifKO mice show morbid feeding, which may result from increased excitability of NPY/Agrp-expressing neurons in the ARC nucleus, resembling our findings in CA1 hippocampal neurons. As a result, Dicer-ifKO cannot correctly adapt to the cycling fluctuations associated with the physiological alternation of orexigenic and anorexigenic neuropeptide-mediated stimuli that regulate the hunger/satiety cycle. Interestingly, circadian rhythm, another typical example of cycling feedback loop, is also altered by Dicer loss (Chen et al. 2013).

The results of our genomic screens were also in line with a subtle role of miRNAs in regulating gene expression. Most miRNAs are expressed from polycistronic clusters that are co-regulated and from which each miRNA is predicted to regulate up to hundreds of targets (often functionally related) with a large redundancy among miRNAs (Gurtan and Sharp 2013; Hausser and Zavolan 2014). Consistent with this view, our analyses revealed a broad impairment in mature miRNA production and a large enrichment of predicted targets among affected mRNAs. However, transcriptomics data exposed surprisingly modest changes. Even the most affected genes, except for a few changes related to the Cre-recombinase transgene, presented changes with lower than a 20% variation in transcript level.

In addition to clarifying the role of Dicer and the miRNA system in mature neurons, our findings have important clinical implications. Thus, the discovery of a link between obesity and Dicer/miRNAs provides a new target for therapeutic intervention in feeding disorders, a leading medical problem in many countries. Furthermore, reduced levels of Dicer and/or mature miRNAs have been reported in patients with temporal lobe epilepsy and in mice subjected to experimentally induced epilepsy (McKiernan et al. 2012), which may relate to the increased neuronal excitability observed in Dicer mutants. Finally, our results indicating that the absence of Dicer is initially well tolerated but has dramatic consequences in the long term could support a role for miRNA dysregulation in the etiology and progression of slow neurodegenerative conditions that are associated with neuronal overactivation and cellular stress, such as Alzheimer's disease (Palop et al. 2007; Lau et al. 2013).

Experimental Methods

Animals and Treatments

CaMKIIa-creERT2 (Erdmann et al. 2007) and Dicer1$^{f/f}$ (Harfe et al. 2005) strains were crossed to produce Dicer-ifKO mice. Both parental strains are available at public reservoirs (EMMA EM:02125 and Jackson Labs #6366, respectively) and are maintained in a C57BL/6J genetic background. *Dicer1* recombination was induced by 5 intragastrical administrations of tamoxifen (Sigma Aldrich, 20 mg/mL dissolved in corn oil) on alternate days. In all of our experiments, we used littermate Dicer1$^{f/f}$ mice lacking the Cre recombinase transgene and treated with tamoxifen as controls. The use of male and female mice was balanced between the genotypes. KA (Tocris, 15 mg/kg) and ghrelin (Abcam, 1 mg/kg) were dissolved in saline and administered by intraperitoneal injection. Mice were maintained according to animal care standards established by the European Union. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

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RNA-seq Screens and Functional Genomics Analyses

For RNA-seq, total hippocampal RNA was extracted with TRI reagent (Sigma), treated with DNase I (Qiagen) and precipitated using potassium acetate. Equal amounts of purified total RNA from 3 adult males (3-month-old, sacrificed 3 weeks after tamoxifen administration) were pooled in each sample. DNA libraries for small RNAs and mRNAs were produced and sequenced using a HiSeq-2500 apparatus (Illumina, service provided by Fasteris S.L.) according to the manufacturer's instructions. For mRNA profiling, the size of the library was 13–14 million sequence reads per sample. Sequenced reads were trimmed for adaptor sequence and masked for low-complexity or low-quality sequence, then mapped to the mouse genome (build GRCm38/mm10) using Bowtie 2 v2.0.5 and TopHAT v2.0.6. SAM/BAM files were further processed using SAMtools v0.1.18. Read count quantitation was performed using SeqMonk (0.26.0). Normalization of read counts and differential expression analysis between genotypes was carried out using the DESeq2 R package from Bioconductor (Release 2.13) (Anders and Huber 2010). Differential analysis output was filtered by FDR threshold (adj. $P < 0.1$).

For small RNA profiling, we mapped 17–22 million sequence reads per sample to the mouse genome (build GRCm38/mm10) and quantified 109,108 annotated features (miRNAs, 2035; miscRNA, 491; mRNA 76938; rRNA, 341; snoRNA, 1602; snRNA, 1431; tRNAs, 26248; tRNA, 22). Sequenced reads were trimmed for adaptor sequence and masked for low-complexity or low-quality sequence, then filtered by length (insert size retained: 18–50 bp) and mapped to the mouse genome (build GRCm38/mm10) using Burrows-Wheeler Alignment Tool (BWA) v.0.5.9 (Li and Durbin 2010). SAM/BAM files were further processed using SAMtools v0.1.18. Read count quantitation was performed using SeqMonk (0.26.0). Normalization of read counts and differential expression analysis between genotypes was carried out using the DESeq2 R package from Bioconductor (Release 2.13). Differential analysis output was filtered by FDR threshold (adj. $P < 0.1$).

Functional genomics enrichment analyses were performed using *WebGestalt* (for phenotype and target enrichment analyses) (Wang et al. 2013) and DAVID (for all other enrichment analyses presented in Supplementary Table 4) (Huang et al. 2009). RNA-seq data are accessible through the Gene Expression Omnibus database using the accession number GSE60263.

PCR and RT-qPCR

For genomic DNA extraction, mice were euthanized by cervical dislocation and dissected tissues were treated with 25 mM NaOH and 0.2 mM EDTA (pH 12) for 20 min at 95°C and then buffered with an equal volume of 1 M Tris-HCl (pH 5). PCR was performed for 30 cycles (1 min at 95°C, 1 min at 54°C, 1 min at 72°C) using GoTaq DNA polymerase (Promega). For RNA extraction, dissected tissues were treated with RNAlater (Qiagen) and total RNA was extracted using the MirVANA kit (Ambion). Reverse transcription was performed using the RevertAid First-Strand cDNA synthesis kit (Fermentas) and RT–qPCR was carried out using the 5× PyroTaq EvaGreen qPCR Mix Plus (Cultek Molecular Bioline) in an Applied Biosystems 7300 real-time PCR unit. Each independent sample was assayed in duplicate and normalized for GAPDH levels. All primer sequences are presented in Supplementary Table 5.

Immunological and Histological Methods

For Nissl staining, brain slices were incubated with cresyl violet for 30 min and then dipped briefly in distilled water, washed briefly in 0.1% acetate in 95% ethanol, followed by 95% and 100% ethanol washes, dipped in xylene twice for 3 min, and quick mounted with Neo-Mount. Thickness measurements in Nissl staining preparations were performed using NIH ImageJ software from images obtained using an epifluorescence microscope (Leica DFC300FX); 2 coronal brain slices from each animal were examined. For IHC, mice were anesthetized with a ketamine/xylazine mixture and perfused with paraformaldehyde (4% in 0.1 M phosphate buffer); brains were postfixed overnight and cut on a vibratome. Immunostaining was performed on 50-µm free-floating slices using the antibodies listed in Supplementary Table 6. For Dicer immunostaining, sections were incubated at 80°C in preheated sodium citrate buffer (pH 6.0) containing 0.05% Triton X-100 for 30 min. Brain slices were washed with phosphate buffered saline (PBS), permeabilized with PBS containing 0.25% Triton X-100, and incubated with the primary antibodies. For diaminobenzidine (Sigma) immunostaining, sections were pretreated with 0.6% H_2O_2 to block endogenous peroxidases. Slices were incubated in the primary antibody solution overnight at 4°C and the secondary antibody incubation was performed at room temperature for 1–3 h. For immunofluorescence, sections were counterstained with 1 nM DAPI (Invitrogen). For quantification of Fos-positive cells, 2 sagittal slices from each hippocampus were examined. Multiarea images were obtained with a confocal laser scanning microscope (Olympus Fluoview FV1200) in multiple stacks and the results of the quantification were averaged.

Behavioral Analysis

Dicer-ifKO mice and control littermate males were tested in the OF and FC tasks. A balanced group of male and female mice (50:50) was evaluated in the EPM task. The OF and EPM tasks were conducted as previously described (Viosca et al. 2009). For training in FC, mice were placed in the conditioning chamber (Panlab S.L., Barcelona, Spain) for 2 min before the onset of 2 foot shocks of 0.6 mA with an interval of 2 s; after an additional 30 s in the chamber, mice were returned to their home cage. Contextual FC was assessed 24 h, 72 h, 1 week, and 2 weeks later by scoring freezing behavior in the same context in which mice were trained using a piezoelectric accelerometer that transduced animal movements and *Freezing* software (Panlab S.L.).

The transcriptional response to novelty was evaluated in an independent group of Dicer-ifKO and control littermate males. Mice were individually placed in 50 × 50 cm^2 OF chambers (170 lux on the floor) containing sawdust, different objects, and spatial references for 1 h and immediately sacrificed or returned to their home cage for 1 h before RNA extraction or animal perfusion.

Seizure induction was evaluated in female mice using a modified Racine Scale: 0 = no response; 1 = facial automatism with twitching of the ears and whiskers; 2 = convulsive waves propagating along the axis of the trunk, immobility and head bobbing; 3 = myoclonic convulsions without rearing, Straub's tail, rigid posture; 4 = clonic convulsion with loss of posture (1–2 episodes) and rearing; 5 = repeated, forceful, clonic-tonic, or lethal convulsions.

For weight measurements, mice were housed in a normal cage with water and food available ad libitum in a 12 h light/dark cycle and weighed every day of tamoxifen administration and then every week after the first administration. For food intake experiments, mice were housed individually for 48 h before experimentation to minimize the effect of the initial stress associated with individual housing. Subsequently, food intake and weight gain were measured every day for 2 h after the start of the light phase. In food deprivation experiments, mice were deprived of food for 15 h. To evaluate food intake in response to orexigenic stimuli, mice received an intraperitoneal injection of

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ghrelin or saline in a randomized order during the light phase, and food intake was monitored over the following 5 h.

Electrophysiology

Whole cell recordings were performed in acute slices of DicerifKO and control littermates aged 6–8 weeks. Mice were sacrificed by cervical dislocation and coronal slices that included the hippocampus (300 μm) were cut in oxygenated (95% O_2/5% CO_2) ice-cold-modified artificial CSF containing (in mM): NaCl 124, KCl 2.5, $NaHCO_3$ 26, NaH_2PO_4 1.25, $MgCl_2$, 2.5, $CaCl_2$ 0.5, and glucose 10. After a recovery period of 30 min at 37°C, slices were maintained at room temperature in standard ACSF containing (in mM): NaCl 124, KCl 2.5, $NaHCO_3$ 26, NaH_2PO_4 1.25, $MgCl_2$, 1, $CaCl_2$ 2, and glucose 10, until use. For recordings, slices were transferred to the recording chamber and superfused with ACSF at 31–33°C. Whole cell recordings were made from CA1 pyramidal neurons using infrared differential interference video microscopy (E600FN, Nikon, Tokyo, Japan). Patch-clamp pipettes were filled with intracellular solution containing (in mM): $KMeSO_4$ 135, NaCl 8, HEPES 10, Mg_2ATP 2, and Na3GTP 0.3 (pH 7.3 osmolarity 295 mOsm/kg). Access resistance was 8–15 MΩ and was monitored throughout the experiment. Experiments were discarded if the series resistance changed by more than 20% during the course of the experiment. Liquid junction potential was −4 mV, and was not corrected. Signals were recorded using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) low-pass filtered at 6 kHz and digitized at 20 kHz (Digidata 1320A, Molecular Devices). All cells described in this study had a membrane potential more negative than −50 mV. Electrical activity was recorded using AxoGraph × 1.0 (AxoGraph Scientific, Sydney, Australia) and analyzed using Clampfit 10.1 (Molecular Devices Corporation). To investigate the firing properties of neurons, 18 current injection steps (500 ms) were applied from −250 to + 500 pA in 50 pA increments from a holding potential of −65 mV. Passive membrane properties were measured at resting membrane potential. Only neurons with at least 3 action potentials during the depolarizing pulse were used for analysis of the mAHP. mAHP was measured at the negative peak in the 20–50 ms range after each action potential. In all electrophysiological experiments, "n" indicates the number of cells, and the data are given as mean ± error. Student's *t*-test was used for statistical data analysis in Sigmastat 3.5 (SYSTAT software). Experimenter was blind to mice genotype.

Authors' Contributions

A.F. and M.S. designed and performed behavioral and cellular and molecular biology experiments and analyzed the data. V.R. conducted and analyzed the electrophysiological recordings. J.L.A. helped in the preparation of NGS experiments and performed most of the bioinformatics analyses. E.G.B. directed and analyzed electrophysiological experiments. A.B. directed the work, designed experiments, analyzed data, and wrote the manuscript. All authors commented on the manuscript.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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Authors' contribution

Some of the experiments presented in this article were performed in collaboration with other members of my lab and the IN that are co-authors of the paper.

I designed and performed behavioral, cellular and molecular biology experiments analyzed and interpreted the data and helped in the design of electrophysiological experiments. José Lopez-Atalaya helped in the preparation and analysis of NGS experiments, Victor Rovira conducted and analyzed the electrophysiological recordings and Marilyn Scandaglia performed EPM experiments.

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DISCUSSION

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The manuscript presented in the Result section includes the discussion of the main findings achieved in this thesis. I will discuss here in greater detail some aspects of our research that were not covered in the manuscript due to space limitations. I will also discuss some new findings in this field that were published during or after the submission of our article.

1. RNAseq screens and functional genomics identify the main biological processes impaired by disrupting miRNA maturation.

As mentioned in the introduction, cell death is the most common consequence of Dicer ablation, observed in almost all the different models investigated so far with few exceptions, such as the mouse olfactory system that does not show apparent variations in cell number and viability even several months after gene ablation (Choi et al. 2008). One of the first goals of our study was the identification of a temporal window in which Dicer ablation and miRNA levels reduction are detected in adult forebrain neurons in the absence of neurodegeneration and gross hippocampal neuronal circuit alteration. Once we defined an appropriate time window for our experiments, we conducted two complementary differential screens for changes in small RNA species and mRNAs in order to identify specific molecules and biological processes affected by the loss of Dicer and the miRNA system. Although previous experiments in other mouse strains lacking Dicer in forebrain neurons have also reported changes in miRNAs (Babiarz et al. 2011) and their potential targets (Dorval et al. 2012), those screens were not conducted in parallel and did not discriminate against indirect changes related to ongoing neurodegeneration. Our experiments, conducted at a time point in which neither degeneration nor gliosis were observed, revealed a striking array of phenotypes that are consistent with an increase in neuronal responsiveness. Working in absence of any signs of neurodegeneration in hippocampus, one of the most injury-sensitive brain structures, therefore, allowed us to conclude that phenomena, such as the enhanced seizures or reduced memory extinction, were

primary consequences of Dicer ablation and not a side effect resulting from the neurodegenerative process.

Importantly, our genomic screens revealed for the first time several interesting features. As expected, Dicer ablation affected specifically miRNA species if compared with other small RNAs, such as snoRNAs and tRNAs, that resulted completely unaffected. Although we observed a general trend towards downregulation, only about 10% of total miRNAs detected in the hippocampus resulted significantly reduced according to the threshold set in our genomic screen. Several factors may account for this result and explain why some miRNAs are more affected than others. First, the existence of non-canonical pathways for miRNA biogenesis has been recently demonstrated (Miyoshi et al. 2010). Dicer-independent pathways could be responsible for the production of miR-320 and miR-451 that are not affected in our model but also of other Dicer-independent miRNAs not yet identified. For instance, MiR-101a, the only miRNA upregulated in our Dicer-ifKO mice, is a candidate miRNA to be produced bypassing Dicer-mediated cleavage. Second, the fact that many miRNAs levels were not significantly reduced could be explicated by the reported high stability of miRNAs (Gantier et al. 2011) and the presence of residual amounts of Dicer at this relatively early time after recombination. In these conditions, the biogenesis of activity-induced miRNAs is presumably strongly compromised. Third, our Cre-mediated Dicer ablation occurs specifically in adult glutamatergic forebrain neurons and a part of detected miRNAs may originate from different hippocampal cells, like astrocytes or glia.

Many miRNAs with important functions in neuron physiology were found differentially expressed, including miR-134 and miR-138. Interestingly, in the mir132/212 bicistron, only miR-212 was found to be significantly reduced in our screen whereas miR-132 was apparently not affected. According with this result, pre-miR-212 levels increase in Dicer-ifKO in basal condition without changes in pre-miR-132, suggesting that in our model some pre-miRNAs may accumulate in the cytoplasm as a result of interfering with their maturation to miRNA. In addition, the changes observed

in pre-miR-212 levels in basal condition might suggest a possible role for hippocampal miR-212 in that condition in spite of its more studied activity-dependent induction and function. Interestingly, although both 5p and 3p forms are reduced, we observed a significant bias towards 3p miRNAs, which for long time were thought to be inactive RNAs with the unique function of passenger strand for the 5p form. The slowly reduction of the 5p miRNA form could be explained by their association with Ago in the RISC complex and/or other accessory proteins that could protect these small RNA from rapid degradation. Nevertheless, due to the recent discovery of functional activities for some 3p miRNAs, a possible implication for these miRNAs in the observed phenotype could not be excluded.

The analysis of the second RNA-seq based screen for larger poly-adenylated RNAs revealed important data about miRNA targets. Except for changes related to the expression of the Cre-recombinase transgene, we only observed relatively modest changes in transcript levels. This may be due to the subtle role of miRNA in regulating gene expression by inhibiting their translation rather than reducing mRNA levels. For this reason, it is possible that the modest changes in transcript levels led to larger variations at the protein level. Notably, when we focused specifically on significantly up-regulated transcripts, we detected a large enrichment of predicted targets among affected mRNAs with a strong overlap (> 50%) between the predicted affected miRNAs according to *WebGestalt* and the differentially expressed miRNAs identified in our small RNA-seq screen, supporting the identification of direct miRNA targets. Such coincidence was not observed in the case of down-regulated mRNAs. In fact, crossing the data from our two RNA-seq based screens, we found several example of upregulated mRNAs that are predicted been targets of many down-regulated miRNAs. This is the case of the ATP-dependent RNA helicase DDX3X (Gene ID: 13205), the predicted target of >30 seed families, the thymoma viral proto-oncogene 3 (Akt3) (Gene ID: 23797), target of 12 seed families, and *Eif2c1* (or Ago1) (Gene ID: 236511), target of ~30 seed families. On the other hand, while *Cnot1* (Gene ID: 234594) is the

predicted target of a unique down-regulated miRNA, miR520d, the two validated up-regulated mRNAs for Tnrc6c (Gene ID: 217351) and *Cacng* (Gene ID: 12299) are not found in our miRNA target enrichment analysis, suggesting an indirect role for miRNA system in their up-regulations.

Analyzing the differentially expressed mRNAs found in Dicer-ifKO, we observed important functional and phenotypical associations. A first group of upregulated transcripts, including Ago1, Adarb1, Tnrc6c and Cnot1, is related to a possible compensatory response to Dicer loss that tries to potentiate alternative miRNA biogenesis pathways and/or RNA-mediated silencing processes. Intriguingly, most upregulated genes were related to *Neurotrophin signaling pathway*, *Synapse* and *Phosphorylation and Protein kinase* suggesting possible alteration in neuronal physiology and responsiveness. According to this view, enrichment for phenotype-related genes revealed significant associations with *Abnormal synaptic transmission*, *Abnormal synaptic plasticity* and *Seizures*. Notably, more than a dozen genes related to seizures were differentially expressed in the hippocampus of Dicer-ifKO mice, including *Cacna1g*, *Akt3*, *Kcnt1*, *Hcn2* and *Bsn*. Taken together, these data support the hypothesis that the miRNA system directly exerts a negative control on synaptic function and responsiveness in order to avoid alteration that could lead to pathological condition derived from neuronal over-activation.

In addition, the identification of upregulated mRNAs and downregulated miRNAs retrieved in our genomic screens provide new subjects of study for future projects and open a series of intriguing avenues to investigate in greater depth their mechanism of action. The identification of a precise regulation mechanism may be particularly important in the case of genes related to epilepsy above mentioned, to develop new strategies in the treatment and/or prevention of seizures. Note that our screens were conducted in basal conditions and thereby all the identified mRNAs are likely targets of constitutive expressed miRNAs. Future genomic screens for the identification of changes in miRNA and mRNA levels in response to stimuli using

conditional and inducible Dicer-KO mouse lines may also address important new findings in the study of activity-induced miRNAs and their targets.

2. Dicer ablation-mediated miRNAs reduction affects the responsiveness of hippocampal neurons

As outlined in the introduction, miRNAs have been implicated in a broad range of physiological and pathological processes both during embryonic development and in adults (Ameres and Zamore 2013, Gurtan and Sharp 2013, Hausser and Zavolan 2014), where they are able to control specifically synaptic function and remodeling, but their specific role and mode of action are still under debate.

Two non-exclusive views have emerged from the rapid progress in this area of research during the last decade. On the one hand, numerous studies have focused on binary miRNA–target interactions depicting relatively simple molecular cascades that contrast with the large number of potential targets for each miRNA predicted *in silico*. On the other hand, computational models and systems biology analyses propose that the miRNA system, as a whole, may confer robustness to biological processes by reinforcing and buffering transcriptional programs (Osella et al. 2011, Pelaez and Carthew 2012). Given the small reduction in protein output (<2-fold) in response to binary interactions between individual miRNAs and their targets and the common ability of compensate this loss in many miRNA loss-of-function models, this second hypothesis affirms that several miRNAs can co-target the same mRNA causing a bigger reduction at the protein level due to a cumulative effect or be players in feedback control loop amplifying their action. In this view, miRNAs could cooperate regulating the same pathway showing functional redundancy. These functional associated miRNAs could belong to the same seed family or only share the same gene or set of genes.

Based on these two models, the phenotypes observed in Dicer-ifKO mice may originate from the dysregulation of a single or a few miRNA-target pairs, as recently proposed in the case of hyperphagia (Vinnikov et al. 2014), or be the result of an overall decrease in miRNA abundance and reduced robustness according to the buffering role discussed above. In fact, the buffering role of miRNAs in mature neuronal circuits is likely to primarily influence biological processes that rely on acute transcriptional waves, such as those induced in neurons in response to stimuli, and on cycling feedback loops, such as feeding behavior. In these cases, miRNAs could act as rheostats to adjust and optimize the mRNA output previously generated by transcription and pre-mRNA processing. We believe that the phenotypes observed in our comprehensive characterization are consistent with the robustness model, although the effect of binary regulatory interaction miRNA:mRNA cannot be excluded. Interestingly, we reported several novel hippocampal-related alterations associated with early Dicer loss indicating that neuronal responsiveness to transient incoming signals was boosted as a result of blocking miRNA biogenesis. We reported that mice lacking Dicer and, as consequence, miRNA system are more sensitive to kainate-induced seizures if compared with their control littermates. In fact, the same doses of this compound induce more severe seizures in mutants than in controls leading, in some cases, to death. In addition, we observed that the induction of IEGs in mutants is stronger in comparison with controls. In order to test if the stronger IEGs induction was the consequence of Dicer loss or only an effect associated to stronger seizures, we also studied their induction in response to novelty exposure. Similarly to the previously response to kainate administration, Dicer-ifKO mice showed a more robust induction of IEGs. Confirming the augmented neuronal responsiveness, we also observed an increase in the excitability of CA1 pyramidal neurons.

The increased levels of IEG induction after neuronal stimulation (by kainate or novelty) could reflect the inability of the neurons to adapt their response to fluctuations in gene expression.)MiRNAs could be important players in the fine-tuning regulation of

physiological IEG levels in response to kainate or novelty. In fact, as mentioned in the introduction, miRNAs are able to induce a stronger repression of mRNA levels when the target transcription stops, like in the case of activity-induced mRNAs, but not for constitutive expressed ones, in which repression is weaker. Interestingly, other studies have also revealed miRNA-dependent phenotypes that were only manifested upon the application of certain stimuli or stresses (Miska et al. 2007, Li et al. 2009, Herranz and Cohen 2010). Importantly, miRNA system could be responsible for the maintaining of correct IEG levels in response to stimuli avoiding an exaggerated neuronal activation leading to neuronal over-excitation and, upon stimulation, seizures. In addition and closely related to enhanced responsiveness, we confirmed the paradoxical memory enhancement phenotype associated with early gene ablation (Konopka et al. 2010) and demonstrated that formed memory was not only stronger but also more resistant to extinction. Similarly to IEG induction, the memory response to fear appears excessive with Dicer-ifKO mice in freezing almost all the time spent in the fear-associated context showing a maximum response, just present after 24 hours after the conditioning phase, that is maintained unaltered in later time points, such as 2 weeks after the conditioning.

All these phenotypical observations suggest the inability of hippocampal neurons to correctly respond to stimuli due to the impossibility to control activity-induced gene expression in a physiological range and, as a consequence, the hippocampal-dependent mouse behavior. At later time points, hippocampal neuronal death and gliosis are strongly observed leading to the almost complete disappearance of hippocampal structure. Importantly, the enhanced responsiveness does not only explain the various early phenotypes but may also explain the slow and progressive neurodegeneration of sensitive structures, such as the hippocampus, observed at later time points.

3. Dicer ablation and subsequent miRNAs reduction in the hypothalamic arcuate nucleus cause hyperphagia and obesity

In parallel to the changes in the hippocampus, Dicer-ifKO mice show morbid obesity associated with hyperphagia, which may result from increased excitability of NPY/Agrp-expressing neurons in the arcuate nucleus, resembling our findings in CA1 hippocampal neurons. In fact, although Dicer loss and the resulting augmented excitability could occur in both NPY/Agrp and POMC neurons, the activity of the orexigenic neuronal population of ARC could block the anorexigenic action of POMC neurons due to the peculiar feedback circuit that characterize this hypothalamic nucleus. In addition, we tested the hyperphagia of Dicer-ifKO in basal condition, with food *ad libitum*, and compared it with the response to food deprivation-mimicking doses of ghrelin at the time point in which we observed the stronger weight gain and the parallel enhanced hippocampal response. We found that basal food intake of Dicer-ifKOs was similar to the food intake in response to food deprivation or ghrelin, namely the maximum response. Interestingly, NPY/Agrp neurons are the unique ARC population expressing ghrelin receptor, suggesting that hyperphagia in these mice is mediated by the hyper-activation of these specific neurons. As a result, Dicer-ifKOs cannot correctly adapt to the cycling fluctuations associated with the physiological alternation of orexigenic and anorexigenic neuropeptide-mediated stimuli that regulate the hunger/satiety cycle because NPY/Agrp neurons appeared permanently activated.

Interestingly, circadian rhythm, another typical example of cycling feedback loop, is also altered by Dicer loss (Chen et al. 2013). Although at later time points in hypothalamus was also observed neurodegeneration and gliosis, many neurons in the ARC continue to be detected, according to another study (Vinnikov et al. 2014). In this case, the absence of neurodegeneration could be explicated by a re-adjustment of the hypothalamic circuit controlling food intake, able to reduce the over-activation of NPY/Agrp neurons and rescue the obese phenotype, as observed in later time points. The mentioned re-setting of the circuit is likely mediated by the hormone leptin that,

produced by adipocytes proportionally to fat store, is able to re-regulate food intake circuitry and adapt its response to the new condition.

Interestingly, in parallel to our research, two other studies have also explored the dysregulation of feeding behavior after Dicer ablation. The first one was published during the preparation of our manuscript and the second was published few months after our study.

In the first study, the group of Witold Konopka reported a similarly transient hyperphagia-induced obese phenotype in Dicer-ifKO mice characterized by a strong increase in fat mass and adipocytes size and demonstrated that the Dicer recombination occurred not only in cortex and hippocampus but also in hypothalamus, identifying ARC as the only region capable to induce obesity (Vinnikov et al. 2014). They also observed a dysregulation in orexigenic/anorexigenic neuropeptides levels, including an increase in NPY protein levels early after recombination, suggesting its important role triggering obesity due to a strong increase in food intake. Circulating leptin levels were also found increased, likely in response to the elevated fat mass. In addition, they identify the over-activation of the PI3K-Akt-mTor signaling pathway in ARC neurons as a cause for hyperphagic obesity that could be attenuated by the infusion of miR-103, the only highly expressed hypothalamic miRNA able to regulate mTor pathway.

In the second study by Mang and colleagues, a severe and transient obesity induction was observed associated with hyperphagia and reduced metabolic rate (Mang et al. 2015). In particular, they observed a reduction of basal metabolism and locomotor activity in Dicer-ifKO mice along with the lack of typical food-seeking behavior in response to 15 hours of fasting. In contrast with our results and the aforementioned study by Vinnikov et al., they have postulated the cortex as the brain area responsible for the restoration of body weight, hypothesizing that adult

neurogenesis and the formation of new neuronal connection could be responsible for the reversion of obesity.

Overall, the three studies report a similar dynamics for the development of hyperphagia-induced obesity in Dicer1 cKO mice, which is characterized by a strong and rapid increase in weight gain and food intake followed by a restoring of normal mouse weight, confirming the robustness and the high reproducibility of this phenotype. These three studies together provides new insights into food intake alteration leading obesity onset and the important role of miRNA system in the regulation of feeding behavior.

However, except for reporting the same obese phenotype, the three studies show important differences in the interpretation of the results. The first difference, perhaps the more evident, is the choice of Mang and colleagues to postulate the cortex as the brain region responsible for the dysregulation of food intake and energy metabolism, in contrast with our study and the paper of Vinnikov et al. demonstrating the impact of miRNAs loss in hypothalamus and the implication of arcuate nucleus in the generation of the observed phenotype. Nevertheless, the possible involvement of cortex and hippocampus, two brain regions important in the regulation of the motivational aspect of the feeding, could not be excluded, albeit it needs to be demonstrated. Despite of the aforementioned agreement about the arcuate nucleus, the study from the Konopka's lab differs from ours in the postulated underlying mechanism. Vinnikov et al. postulate the downregulation of a single miRNA, miR-103, as responsible for the metabolic alterations. Supporting this view overexpression of miR-103 could partially rescue the observed phenotype. This approach is clearly in line with the hypothesis that miRNAs act trough binary interactions with their targets. Nevertheless the interesting results obtained, they found only one miRNA, the miR-103, able to attenuate the obesity but not to rescue it. In our study, we favored the notion of considering the miRNAs like a system that, as a whole, is responsible for the

precise regulation of neuronal responsiveness and cycling activation. Disrupting this system in the ARC nucleus altered food intake and energy metabolism and induced obesity in Dicer-ifKO mice. In our view, miR-103 could rescue the excessive food intake and obesity triggered together with other unidentified miRNAs that could potentiate its action probably by co-targeting. However, miR-103 was not found downregulated in our screen for small RNA, supporting a specific role for this miRNA in the hypothalamus.

In addition and according to the hypothesis considering miRNA system as a whole, we focusing on the implication of miRNA system loss in ARC and demonstrated indirectly, using ghrelin administration, the role for Agrp/NPY neuronal over-activation in the induction of obese phenotype. Our genomic, electrophysiological and behavioral experiments in the hippocampus provide additional evidences supporting the hypothesis that the lack of miRNA system is associated with neuronal over-activation, thus suggesting a role for these small RNAs in the regulation of neuronal function.

4. Clinical implications of our study

Our results suggest that the Dicer/miRNA system may play a critical role in setting a threshold for neuronal activation in the hippocampus and the hypothalamus, two brain regions implicated in the response to environmental and/or hormonal stimuli. MiRNAs may maintain neuronal responses in physiological ranges regulating gene expression and the associated protein output via tuning interactions that, even if induce only weak changes in basal condition, give rise to stronger and functionally important control of activity-induced targets. The miRNA-mediated regulation is thus crucial for constituting a molecular brake for important activity-induced processes, such as memory and feeding behavior, but also to protect sensitive neurons against over-activation.

In addition to clarifying the physiological role of Dicer and the miRNA system in mature neurons, our findings have important clinical implications. The discovery of a link between obesity and Dicer/miRNAs provides new targets for therapeutic intervention in feeding disorders, a leading medical problem in many countries. Our results clearly demonstrate the association between hypothalamic miRNA dysregulation and the obesity pathogenesis. Future studies are required to specifically identify obesity-related miRNAs and their targets in order to design new therapies against obesity and the associated metabolic conditions.

Furthermore, reduced levels of Dicer and/or mature miRNAs have been reported in patients with temporal lobe epilepsy and in mice subjected to experimentally induced epilepsy (McKiernan et al. 2012a), which may relate to the increased neuronal excitability observed in Dicer mutants. Our results may provide new players for understanding epilepsy pathogenesis and designing novel therapeutical approaches; these include some of the miRNAs found reduced in Dicer-ifKO mice and their upregulated target mRNAs.

Finally, as in previous studies in other conditional KO strains (Davis et al. 2008, Hebert et al. 2010, Konopka et al. 2010), our experiments revealed a requirement for Dicer in long-term survival of forebrain principal neurons. In fact, we show that the slow neurodegeneration of sensitive structures, such as the hippocampus, was associated with gliosis and the expression of excitotoxicity markers (Valor et al. 2010), which again connects to aberrant responsiveness. We believe that our results indicate that the absence of Dicer is initially well tolerated but has dramatic consequences in the long term could support a role for miRNA dysregulation in the etiology and progression of slow neurodegenerative conditions that are associated with neuronal over-activation and cellular stress, such as Alzheimer's disease (Palop et al. 2007, Lau et al. 2013).

In conclusion, in addition to provide new information about the role of miRNAs in neuronal function and responsiveness, our study also generates important start points for future experiments aimed to uncover the mechanism of action of specific miRNA in the regulation of their target levels and developing new strategies to therapeutically approach pathological conditions such as obesity, epilepsy and neurodegeneration.

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CONCLUSIONS

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Conclusions

1. Conditional and inducible Dicer1 ablation in adult glutamatergic forebrain neurons causes progressive neurodegeneration that, in later time points, results in generalized brain gliosis associated with severe neurodegeneration of hippocampal structures, strong cortical atrophy and a marked enlargement of the third ventricle.

2. The detection of a temporal window in which Dicer ablation and miRNA levels reduction are not yet associated with neurodegeneration made possible the performance of two complementary genomic screens aimed to identify the specific molecules and biological processes affected by Dicer/miRNA system loss, avoiding the occluding impact of change in the cellular composition of the tissue.

3. The RNAseq screen for small RNAs demonstrated a selective downregulation of miRNAs, whereas other sncRNA species are spared, and identified a number of miRNAs whose biogenesis seems to be Dicer-independent.

4. The RNAseq-based differential screens for changes in mRNAs identified possible direct targets for miRNAs with important roles in the regulation of neuronal function and responsiveness.

5. Dicer loss is associated with enhanced seizure susceptibility and a stronger induction of immediate early genes (IEGs), such as Fos and Arc.

6. Dicer loss is also associated with enhanced physiological induction of IEGs in response to novelty exposure.

7. The early loss of miRNA biogenesis causes the enhancement of fear associative memory. Notably, the contextual fear memory formed in mutant mice is not only stronger but also more resistant to extinction.

8. Dicer loss causes an increase of the excitability of CA1 pyramidal neurons, which may underlay the different molecular and behavioural phenotypes outlined above.

9. In parallel to the changes in the hippocampus, Dicer-ifKO mice show morbid obesity associated with hyperphagia, which may result from increased excitability of NPY/Agrp-expressing neurons in the arcuate nucleus.

10. Taken together, these results indicate that neuronal responsiveness to transient incoming signals is initially boosted as a result of blocking miRNA biogenesis. We propose that the miRNA system plays a critical role in setting a threshold for neuronal activation in the hippocampus and the hypothalamus, two brain regions implicated in the response to environmental and/or hormonal stimuli, acting like a molecular brake for important activity-induced processes, such as memory and feeding behavior and protecting sensitive neurons against over-activation.

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Conclusiones

1. La ablación de Dicer1 de forma condicional e inducible en las neuronas glutamatérgicas del cerebro anterior en animales adultos provoca una progresiva neurodegeneración que, a largo plazo, resulta in una gliosis generalizada del cerebro asociada a severa neurodegeneración del hipocampo, fuerte atrofia de la corteza y marcado agrandamiento del tercer ventrículo.

2. La identificación de una ventana temporal, en la cual la ablación de Dicer y la reducción de los miRNAs aún no están asociados a neurodegeneración, hace posible la realización de dos experimentos de rastreo genómico complementarios usando la técnica de RNA-Seq para identificar moléculas y procesos específicamente afectados por la pérdida de Dicer/sistema de miRNA, evitando el efecto entorpecedor debido a cambios en la composición celular del tejido.

3. El experimento de RNA-Seq para pequeños RNAs ha mostrado una bajada selectiva de los niveles de miRNAs, mientras los de otros sncRNAs resultan exentos de cambios, y ha identificado un número de miRNAs cuya biogénesis parece ser independiente de Dicer.

4. Los rastreos genómicos diferenciales basado en la técnica de RNA-Seq para mRNAs ha identificado posibles dianas directas de los miRNAs con importantes papeles en la regulación de la función y de la capacidad de respuesta de las neuronas.

5. La pérdida de Dicer está asociada a una exagerada susceptibilidad a epilepsia y a una mayor inducción de genes inmediatos tempranos, por ejemplo Fos y Arc.

6. La pérdida de Dicer está también asociada a una exagerada inducción de genes inmediatos tempranos en respuesta a novedad.

7. La pérdida temprana de la biogénesis de los miRNAs causa un aumento de la memoria asociada al miedo. Notablemente, la memoria contextual asociada al miedo en los mutantes no solo es mayor sino más resistente a la extinción.

8. La pérdida de Dicer causa un aumento de la excitabilidad de las neuronas piramidales de CA1, que podría explicar los diferentes fenotipos moleculares y comportamentales mencionados anteriormente.

9. En paralelo a los cambios en el hipocampo, los ratones Dicer-ifKO muestran obesidad mórbida asociada a hiperfagia, que podría ser el resultado de una aumentada excitabilidad de las neuronas que expresan NPY/Agrp del núcleo arqueado.

10. En conjunto, nuestros resultados indican que la capacidad de respuesta de las neuronas a señales entrantes transitorios es en un primer momento impulsada a consecuencia del bloqueo de la biogénesis de los miRNAs.
    Nuestra hipótesis es que el sistema de los miRNA juegue un papel crítico en el establecimiento de un umbral para la activación neuronal en el hipocampo y en el hipotálamo, dos estructuras cerebrales involucradas en responder a estímulos

ambientales y/o hormonales, actuando como un freno molecular para importantes procesos inducidos por actividad, tales como la memoria y la regulación de la toma de alimentos, protegiendo las neuronas frente a la sobre-activación.

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ANNEX I: Supplemental material Cerebral cortex

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Inventory of Supplemental information

A. Supplemental Figures S1-S3 and legends:

Supplemental Figure S1, related to Figure 1. Dicer ablation causes progressive neurodegeneration.

Supplemental Figure S2, related to Figure 3. RNA-seq profiles in the hippocampus of Dicer-ifKO and control mice.

Supplemental Figure S3, related to Figure 7. Dicer ablation in the arcuate nucleus.

B. Supplemental Tables S1-S6 (in the attached CD):

Supplemental Table S1, related to Figure 2. Differentially expressed miRNAs in the hippocampus of Dicer-ifKO mice.

Supplemental Table S2, related to Figure 3. Differentially expressed mRNAs in the hippocampus of Dicer-ifKO mice.

Supplemental Table S3, related to Figure 3. miRNA target enrichment analysis.

Supplemental Table S4, related to Figure 3. Functional genomics analysis of differentially expressed mRNAs in the hippocampus of Dicer-ifKO mice.

Supplemental Table S5, related to Experimental methods. Primers used in this study.

Supplemental Table S6, related to Experimental methods. Antibodies used in this study.

Supplemental Figure Legends

Supplemental Figure S1, related to Figure 1. Dicer ablation causes progressive neurodegeneration. A. Immunostaining of sagittal brain slices from Dicer-ifKO mice 3 and 12 weeks after tamoxifen treatment using an antibody that recognizes a central domain of Dicer. No reduction of Dicer was observed at early time points using this antibody. **B.** Immunostaining of sagittal brain slices from Dicer-ifKO mice 12 weeks after tamoxifen treatment using an antibody that recognizes the C-terminus of Dicer. **C.** Representative images of Nissl staining in the hippocampus and cortex of control and Dicer-ifKO mice at different time points after tamoxifen treatment. **D.** Quantification of layer thickness in the dentate gyrus, CA3 and CA1 subfields of the hippocampus and in the cortex. **E-F.** RT-qPCR for GFAP (C) and H2-Q7/8/9 (D) transcripts in RNA samples from control and Dicer-ifKO mice extracted 3 weeks (3w) and 12 weeks (12w) after tamoxifen treatment.

Supplemental Figure S2, related to Figure 3. RNA-seq profiles in the hippocampus of Dicer-ifKO and control mice. A. RNA-seq profile at the *Esr1* locus presenting the accumulation of reads in exon 9 of *Esr1* (orange box) in Dicer-ifKO mice. This exon encodes the fragment of the estrogen receptor fused to the cre recombinase in the creERT2 construct. **B.** RNA-seq profile at the *Cacna1g* locus (orange box). The modest but highly significant increase in the number of counts in Dicer-ifKO mice is indicated. The normalized read counts in all other loci shown in this snapshot were not significantly different between genotypes. Note the very high reproducibility of the profiles between samples.

Supplemental Figure S3. Dicer ablation in the arcuate nucleus. A. High magnification images of immunostaining for Cre recombinase in the arcuate nucleus of Dicer-ifKO and control littermates 3 weeks after tamoxifen administration. **B.** α-GFAP immunostaining in the arcuate nucleus of Dicer-ifKO and control littermates. The number of glial cells is significantly increased in the hippocampus of Dicer-ifKO mice 12 weeks after tamoxifen administration. **C.** Representative images of Nissl staining in the arcuate nucleus and the third ventricle in brain slices of control and Dicer-ifKO mice at different time points after tamoxifen treatment.

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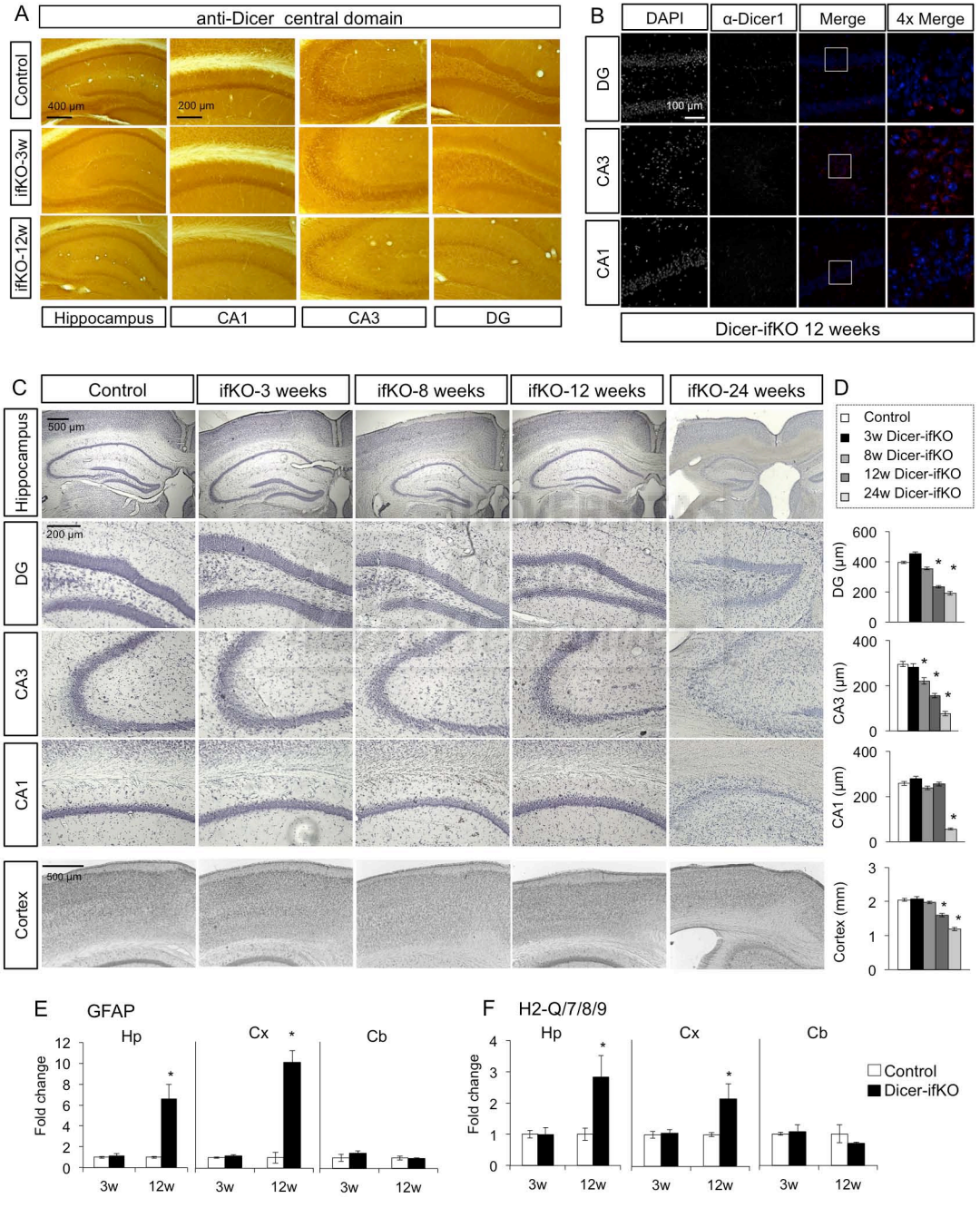
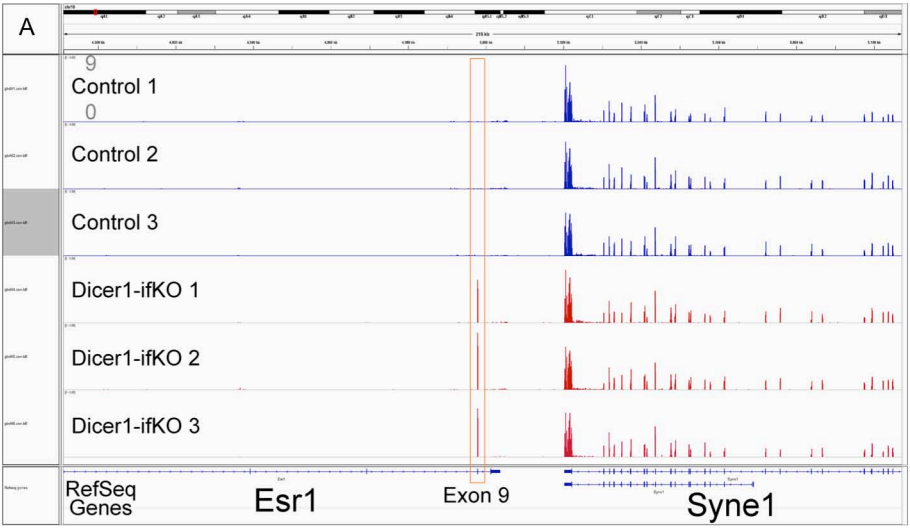
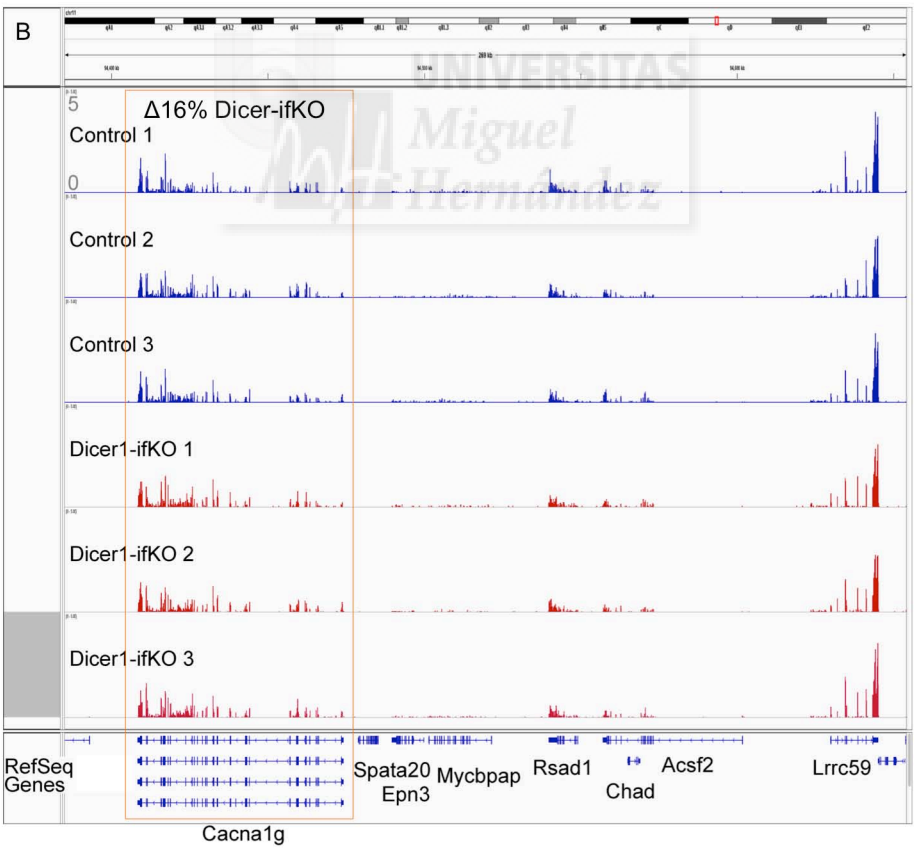
Supplemental Figure S1: Related to Figure 1



Supplemental Figure S2: Related to Figure 3





Supplemental Figure S3: Related to Figure 7