ORIGINAL ARTICLE

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

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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
Additionaly, 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. 2006).

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 dopaminergic 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 deletion 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 miRNAs 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<sup>fl</sup> (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. 1A). The recombination of the Dicer1 locus was confirmed in genomic DNA from the hippocampus and cortex of mutant mice (Fig. 1B), the 2 main brain areas expressing the Cre recombinase (Fig. 1C). In contrast, we did not observe recombination in the cerebellum in which the CaMKIIa promoter is not active (Fig. 1B). Furthermore, exon-specific RT-qPCR assays demonstrated the production of the truncated transcripts in regions showing recombination (Fig. 1D). 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 recombinated transcripts are stable (Fig. 1D, 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. 1E with Supplementary Fig. 1A). This loss of immunoreactivity was particularly prominent in the dentate gyrus and CA1 subfields (Fig. 1E and Supplementary Fig. 1B).

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. 1F and Supplementary Fig. 1C). 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. 1F and Supplementary Fig. 1C,D). The thickness of cortical layers was also significantly reduced (Supplementary Fig. 1C,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. 1E,F). Furthermore, immunostaining against the glial marker GFAP confirmed the neurodegeneration and increased gliosis at late time points after recombination (Fig. 1G). 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 6062 of 1900 after eliminating gene duplications) are expressed in hippocampal tissue (Fig. 2A).

As expected, the screen revealed a specific impact on miRNA biogenesis, whereas all other species of small noncoding RNAs were spared (Fig. 2B,C). Note that by selecting transcripts smaller
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 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.
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. 2D). However, there was a strong overall trend toward lower miRNA levels in Dicer mutants (Fig. 2E). Approximately 10% of the expressed miRNAs were significantly reduced (adj. \( P < 0.1 \)) in Dicer-ifKO mice 3 weeks after triggering gene ablation.
(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-iKO 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-iKO 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 spinoogenesis (Schratt et al. 2006; Siegel et al. 2009), miR-124 that constrains synaptic plasticity in Aplysia neurons (Rajaseethapathy 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-iKO mice. Thus, the 3 genes contained in the BAC bearing the CaMKIIα-creERT2 transgene (Arsl, CamK2a, and Slc6a7) were upregulated in Dicer-iKO 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 intrinsic 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-iKO mice, we find several genes encoding key components of the RNA-induced silencing complex (RISC) that are downstream of Dicer, such as Eff2c1 (aka Argo1, 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-iKO 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-iKO mice, such as the aforementioned Eff2c1, the intellectual disability-associated genes Srgap3 and Sclc6a8, 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-iKO 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 10⁻⁸), which were associated with more than 20% of differentially expressed genes. This prominence was observed in both downregulated and upregulated genes (P = 10⁻⁷). 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 × 10⁻⁷), “Abnormal synaptic plasticity” (adj. P = 2 × 10⁻⁴), and “Seizures” (adj. P = 3 × 10⁻⁴). Notably, more than a dozen genes related to seizures were differentially expressed in the hippocampus of Dicer-iKO mice, including Cacna1g, Akt3,
Kcnt1, Hcn2, and Bsn (Supplementary Fig. 2B). For instance, the elevation of the α1C-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.
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. 4L). 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 Caun1g 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 (Panselov 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, F1,16 genotype = 17.11, P < 0.001, F2,32 time × 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. S3A). 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,
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...
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. 7F). Interestingly, our transcriptomics screen, although conducted in the hippocampus,

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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.
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
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 excitation 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 miRNAs. 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 Dicer1fl/fl (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 intragastric administrations of tamoxifen (Sigma Aldrich, 20 mg/ml dissolved in corn oil) on alternate days. In all of our experiments, we used littermate Dicer1fl/fl 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.
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 reads per sample to the mouse genome (build GRCm38/mm10) and quantified 109,108 annotated features (miRNAs, 2035; microRNA, 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 GoTag 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 ReverTra Aid First-Strand cDNA synthesis kit (Fermentas) and RT-qPCR was carried out using the SYBR Green qPCR Mix Plus (Cultek Molecular Bio-line) 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 0.5% 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% H2O2 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-kO 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-kO and control littermate males. Mice were individually placed in 50 × 50 cm2 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 orogenic stimuli, mice received an intraperitoneal injection of...
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 Dicer−/− mice 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% O2/5% CO2) ice-cold-modified artificial CSF containing (in mM): NaCl 124, KCl 2.5, NaHCO3 26, NaH2PO4 1.25, MgCl2 1.5, CaCl2 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, NaHCO3 26, NaH2PO4 1.25, MgCl2, 1, CaCl2 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): KMeSO4 135, NaCl 8, HEPES 10, Mg2ATP 2, and Na3GTP 0.3 (pH 7.3 osmolarity 295 mOsm/kg). Access resistance was 8–15 MΩ and was monitored throughout the experiment. 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 mouse 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/.

**Funding**

A.F. held a doctoral fellowship funded by the Consolider programme (grant number CSD2007-00023). Research at A.B.’s laboratory is supported by a grant from the Spanish MINECO (SAF2011-22855), Prometeo/2012/005 from the Generalitat Valenciana and a NARSAD Independent Investigator Grant from the Brain & Behavior Research Foundation. Research at E.G.B.’s laboratory is supported by a MINECO grant (BFU2011-27326). The Instituto de Neurociencias is a “Centre of Excellence Severo Ochoa.”

**Notes**

The authors thank Eloisa Herrera, Witold Konopka, and members of Barco’s laboratory for critical reading of the manuscript. Conflict of Interest: None declared.

**References**


