Genetic ablation of *Dicer* in adult forebrain neurons results in abnormal tau hyperphosphorylation and neurodegeneration

Sébastien S. Hébert1,2,3,∗, Aikaterini S. Papadopoulou3,4, Pascal Smith1,2, Marie-Christine Galas6,7, Emmanuel Planel1,2, Asli N. Silahtaroglu5, Nicolas Sergeant6,7, Luc Buée6,7 and Bart De Strooper3,4,∗,

1Centre de Recherche du CHUQ (CHUL), Axe Neurosciences, Québec, Canada, 2Département de Psychiatrie et de Neurosciences, Université Laval, Faculté de Médecine, Québec, Canada, 3Department of Molecular and Developmental Genetics, VIB, Leuven, Belgium, 4Center for Human Genetics, K.U. Leuven and 5Wilhelm Johannsen Centre for Functional Genome Research, Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen N, Denmark, 6Faculté de Médecine, Jean-Pierre Aubert Research Centre, Université Lille-Nord de France, UDSDL, Lille, France and 7Inserm, U837, Alzheimer & Tauopathies, Lille, France

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Type III RNase Dicer is responsible for the maturation and function of microRNA (miRNA) molecules in the cell. It is now well-documented that Dicer and the fine-tuning of the miRNA gene network are important for neuronal integrity. However, the underlying mechanisms involved in neuronal death, particularly in the adult brain, remain poorly defined. Here we show that the absence of *Dicer* in the adult forebrain is accompanied by a mixed neurodegenerative phenotype. Although neuronal loss is observed in the hippocampus, cellular shrinkage is predominant in the cortex. Interestingly, neuronal degeneration coincides with the hyperphosphorylation of endogenous tau at several epitopes previously associated with neurofibrillary pathology. Transcriptome analysis of enzymes involved in tau phosphorylation identified ERK1 as one of the candidate kinases responsible for this event *in vivo*. We further demonstrate that miRNAs belonging to the miR-15 family are potent regulators of ERK1 expression in mouse neuronal cells and co-expressed with ERK1/2 *in vivo*. Finally, we show that miR-15a is specifically downregulated in Alzheimer’s disease brain. In summary, these results support the hypothesis that changes in the miRNA network may contribute to a neurodegenerative phenotype by affecting tau phosphorylation.

INTRODUCTION

Dicer is a type III RNase enzyme responsible for the processing of microRNA (miRNA) precursors into mature (functional) miRNA molecules. The latter comprises a class of highly conserved small (~20 nt) non-coding RNAs that bind the 3′-untranslated region (3′-UTR) of target messenger RNAs (mRNAs) and regulate gene expression at the post-transcriptional level (1). This results in translational repression or degradation of target mRNAs. Individual or families of miRNAs can target up to several hundred mRNAs, thus controlling complex gene expression pathways and biological systems (2). It has been documented that miRNA family members may have independent and/or overlapping functions *in vivo* (3).

It is nowadays well-established that the fine-tuning of the miRNA gene network is important for the integrity of neuronal populations (4–8). In spite of this, the phenotypes obtained using distinct conditional *Dicer* knockouts in the brain are quite variable, probably reflecting the different aspects of
neurodegeneration in which miRNAs are involved. Using α-CamKII-Cre transgenic (Tg) mice that express Cre from embryonic day E15.5, Davis et al. (6) observed abundant neuronal apoptosis (activated caspase-3 and TUNEL-positive stainings) in the absence of Dicer at early [postnatal day 0 (P0)] but not late (P15) developmental stages. It is worthy of note that these mice did not survive past 21 days of age. In line with this, it has been shown that the removal of Dicer at later stages of brain development could contribute to abnormal phenotypes (e.g. increased spine length) but not necessarily neuronal loss (9). The fact that neuronal atrophy but no obvious signs of cell death is observed in Dicer-deficient mature striatal neurons is also consistent with this hypothesis (7). It should be mentioned, however, that conditional knockout of Dicer in adult Purkinje cells results in a neurodegenerative phenotype accompanied by a limited number of apoptotic (TUNEL-positive) neurons (4).

Hyper- and abnormally phosphorylated forms of the microtubule-associated protein tau characterize neurofibrillary tangles (NFTs) and are observed in more than 20 neurodegenerative disorders including Alzheimer’s disease (AD) (10–12). Under physiological conditions, tau promotes the assembly and maintenance of microtubules in neurons and its biological activity is regulated by phosphorylation. It is generally thought that disruption of the normal phosphorylation state of tau plays a key role in the development of NFTs and is intrinsically linked to tau pathology and cell death.

Recent evidence points to a role for miRNA-regulated pathways in modulating tau metabolism and toxicity. Indeed, Bilen et al. (13) showed a striking enhancement of tau-mediated toxicity in Drosophila cells when miRNA maturation was suppressed. More recently, miR-128 was shown to modulate BAG2 expression in cultured cells, a co-chaperone potentially involved in tau degradation and aggregation (14). In this study, we tested the hypothesis that loss of miRNA function could trigger neurodegeneration through mechanisms implicating tau hyperphosphorylation, as seen during neurofibrillary degeneration.

RESULTS

Generation and characterization of mice lacking miRNAs in the adult forebrain

In order to investigate the role of neuronal miRNAs in the adult forebrain, we generated α-CamKII-Cre conditional Dicer knockout (cKO) mice. The CamKII-Cre transgenic mouse used in this study expresses Cre recombinase mainly in excitatory neurons of the forebrain from approximately P18, with maximal recombination of loxp-flanked alleles at 6 weeks (15). Loss of Dicer therefore is mainly restricted to the adult cortex and hippocampus.

We first evaluated the biological effects of Dicer loss in our mouse model. Starting at ~9 weeks of age, the Dicer mutant animals displayed signs of hypoactivity, had decreased social interaction and were less sensitive to external (e.g. touch) stimuli when compared with littermate controls. At 11 weeks of age, 60% (n = 16) of Dicer cKO animals had died (Fig. 1A). Consistent with the observations of Davis et al. (6), a subset (6 of 16) of Dicer cKO mice showed progressive signs of ataxia and/or defects in rear limb movement (data not shown). Four out of 16 mutant Dicer mice developed severe tail and/or neck lesions, presumably due to excessive and injurious self-grooming. None of the Dicer cKO animals survived past 14 weeks of age (Fig. 1A). On the other hand, control mice (n ≥ 50) displayed no phenotypic or behavioral abnormalities and survived to adulthood.

To confirm the loss of functional Dicer, miRNA levels were measured by quantitative real-time PCR (qRT-PCR). A significant reduction in brain miRNAs miR-29a, miR-9, miR-134, miR-107 and miR-124 was observed in the cortex of 9-week-old Dicer cKO mice when compared with littermate controls (Supplementary Material, Fig. S1A). The changes in miRNA expression are likely underestimated as these samples are composed of a heterogeneous mixture of brain cells (e.g. glia and other neuronal types) and it is not expected that Cre is expressed in all the cells.

Neurodegeneration and inflammation in Dicer cKO adult forebrain and hippocampus

At ~9–10 weeks of age, a considerable (~25%) reduction in total brain weight was seen in the Dicer cKO compared with littermate control mice (Supplementary Material, Fig. S1B). Complete morphological analysis of the brains of Dicer cKO mice showed a decrease in size of the cortex, hippocampus (Fig. 1B) and, to a lesser degree, cerebellum. The smaller size of the cerebellum might be the consequence of some residual Cre activity in this brain region (6). In the hippocampus of the mutant mice, abundant neuronal loss, particularly in the CA3/4 areas, was evident as shown by stainings with cresyl violet (Fig. 1C) and the neuronal marker NeuN (Supplementary Material, Fig. S1C). In the cortex, a different image was observed. NeuN stainings showed clear signs of cytoplasmic atrophy, but no obvious neuronal loss (Fig. 1D; controls = 100% versus Dicer cKO = 107 ± 17%, n = 3 from each group). The reduction in size, but not in the total cell number, is in line with the decrease in the 48-kDa cytoplasmic (versus 46-kDa nuclear) NeuN variant (16) and the equal amounts of neuronal marker MAP2 (Fig. 1E). No indications of apoptosis using activated caspase-3 as well as fragmented DNA [using 4′,6-diamidino-2-phenylindole (DAPI)] stainings were observed in the different Dicer cKO brain regions (Supplementary Material, Fig. S2).

An increase in the microglial activation marker Iba1 was observed in the hippocampus of 9-week-old Dicer cKO compared with littermate control mice (Fig. 1Fa–d). We could only consistently detect Iba1-positive stainings in the cortex of 12.5-week-old mutants (Fig. 1Fe and f). Similarly, at 12.5 weeks (but not at 9 weeks), an increase in glial fibrillary acidic protein (GFAP)-positive astrocytes was observed in the cortex and hippocampus of mutant Dicer animals (Fig. 1Ga–d). Taken together, these results show that the absence of functional Dicer in the adult forebrain is accompanied by progressive neuronal degeneration as well as increased inflammation and gliosis.

Tau hyperphosphorylation in the absence of Dicer

To analyze the tau phosphorylation levels in the cortex of 9-week-old Dicer cKO mice, endogenous tau proteins were
resolved by two-dimensional (2D) electrophoresis and labeled with antiserum directed against total tau. A shift of all major murine tau isovariants to the acidic side was observed in the Dicer cKO mice when compared with littermate controls (Fig. 2A). Further characterization of tau phosphorylation was performed using a panel of phospho-tau antibodies (Fig. 2B). These eastern blotting experiments confirmed that endogenous tau in the Dicer cKO brains is hyperphosphorylated at several sites, comprising epitope AT8 (Ser202/Thr205), AT180 (Thr231), AT270 (Thr181), AD2/PHF-1.
(Ser396/Ser404), 12E8 (Ser262/Ser356) and the ‘pathological’ S422 (Ser422) (17). The shift in the molecular weight of tau is consistent with its hyperphosphorylation state. Tau1 (dephosphorylated amino acid 189-207) was decreased in the Dicer cKO mice, in line with the hyperphosphorylation of tau at this epitope. The global amount of tau protein was not altered as controlled using the Tau5 (amino acid 210-241) antibody. Moreover, qRT-PCR showed that total tau mRNA was also unchanged between the Dicer KO and control groups (Supplementary Material, Fig. S3A). Notably, similar to the cortex, increases in tau hyperphosphorylation were observed in the hippocampus of Dicer mutants (Supplementary Material, Fig. S3B). In addition, no change in tau phosphorylation was evident in younger (3-7 weeks old) Dicer cKO mice (Supplementary Material, Fig. S3C). Overall, these results show a site-specific and time-dependent hyperphosphorylation of endogenous tau in vivo in the absence of functional Dicer in the adult brain.

Finally, we evaluated the effects of Dicer loss on tau phosphorylation in neuronal cell cultures. To this end, we performed transient knockdown experiments in Neuro2a cells using short hairpin RNAs directed against Dicer mRNA. Seventy-two hours after transfection, we observed a significant increase in endogenous tau phosphorylation (Supplementary Material, Fig. S3D and E) upon Dicer knockdown. We confirmed the downregulation of Dicer in these experimental conditions by western blot (Supplementary Material, Fig. S3F) and qRT-PCR (data not shown).

ERK1 levels are increased in Dicer cKO brains

As it is unclear how Dicer deficiency can lead to tau hyperphosphorylation, we hypothesized that miRNA-regulated pathways could affect the kinases or phosphatases involved in tau phosphorylation. We, therefore, screened for potential candidates using Affymetrix gene expression arrays. We focused on upregulated kinases or downregulated phosphatases involved in tau phosphorylation and set a +1.25-fold change in expression with \( P < 0.05 \) as cutoff values. Among several candidate enzymes (18), these screens identified mitogen-activated protein kinase 3 (MAPK3/ERK1) to be specifically upregulated in the Dicer cKO mice (Supplementary Material, Tables S1 and S2). Although protein phosphatase 2C was significantly downregulated in the Dicer cKO mice, this enzyme was previously shown to be only weakly involved in direct tau dephosphorylation (19). We therefore
focused our work on ERK1. To validate the microarray data, we performed qRT-PCR and western blot analyses. These experiments showed an increase in ERK1 at the mRNA, protein and phosphorylation levels in the Dicer cKO mutant mice (Fig. 2C–E). To a lesser extent, a significant increase in ERK2 phosphorylation (but not protein levels) was also observed.

Other kinases considered to be in vivo responsible for the regulation of tau phosphorylation include GSK-3β and Cdk5 (20,21). GSK-3β activity is regulated negatively by the phosphorylation of serine 9 (Ser9) and positively by phosphorylation of tyrosine 216 (Tyr216). Western blot analysis of equal amounts of protein showed a drastic increase in GSK-3β Ser9 phosphorylation in the cortex of Dicer cKO mice when compared with littermate controls (Fig. 2F). In contrast, no changes at the GSK-3β Tyr216 site as well as in GSK-3β total protein levels were observed in the mutant Dicer mice.

In the brain, Cdk5 is activated by its interaction with the neuron-specific molecule, p35, or the p35-cleaved product p25. Although Cdk5 total protein levels remained unchanged, a reduction in p35 levels without p25 formation was observed in the mutants (Fig. 2G). The PKB/Akt survival pathway is known to be involved in GSK-3β activity. A marked increase in Akt protein levels was detected in the Dicer cKO brains (Fig. 2G), which could explain the observed effects on GSK-3β Ser9. Of notice, Akt and p35 mRNAs were also upregulated and downregulated, respectively, in our microarray analysis (Supplementary Material, Table S1 and data not shown). Taken together, these results suggest that the GSK-3β/cdk5 pathway is downregulated and therefore not mainly involved in tau hyperphosphorylation in the absence of Dicer.

Finally, there were no significant changes at the protein and activity levels of other mitogen-activated protein kinase family members, namely JNK1 and 2, as well as several major tau phosphatases including PP1, PP2B, PP2AA and PP2AC (Supplementary Material, Fig. S4 and data not shown). Overall, we identify ERK1 (and perhaps ERK2) as a candidate effector of tau hyperphosphorylation in vivo in the conditional Dicer KO mice.

**miR-15 family members regulate ERK1 expression**

The increase in ERK1 protein (and mRNA) levels in the Dicer cKO mice prompted us to investigate whether dysregulation of particular miRNAs could be involved in the regulation of ERK1 expression. We used common prediction programs (TargetScan and miRANDA) to search for potential miRNA target sites on the 3′-UTR of ERK1 mRNAs. These screens identified conserved miRNA seed sequences for the miR-15/16/195/322/497 and miR-1/206 families (Supplementary Material, Fig. S5A and B). We focused our work on the miR-15 family, as miR-1/206 family members are mainly expressed in muscle cells and not in the brain (22). Compared with control brains, miR-15a, miR-195, miR-16 and miR-497 expression levels were significantly reduced in 9-week-old Dicer cKO mice, whereas no significant change was observed for miR-15b and miR-322 in these samples (Supplementary Material, Fig. S5C).

We further evaluated the possibility that ERK1 expression could be regulated by miRNA expression. miRNA precursors (pre-miRs) for miR-15a, miR-16, miR-195 and miR-497 were transfected in Neuro2a cells causing a significant (~55–85%) reduction in endogenous ERK1 protein and phosphorylation levels (Fig. 3A and B). As negative control, we used a scrambled miRNA sequence. We also verified that blocking endogenous miR-16, which is highly expressed in neuroblastoma Neuro2a cells (Supplementary Material, Fig. S5D), results in a ~50 and ~250% increase in ERK1 protein and phosphorylation levels, respectively (Fig. 3C). Here, a scrambled miRNA inhibitor was used as a negative control. Of note, a small but significant increase in ERK2 phosphorylation was equally observed in anti-miR-16-treated cells. In these experimental conditions, a significant (~50–90%) reduction in endogenous miR-16, miR-15a, miR-195 and miR-497 was observed (Supplementary Material, Fig. S5E), likely because of the similarities of these miRNA family members and cross-reactivity of the antisense locked nucleic acid (LNA) probes. These loss of function experiments in cells demonstrate at the endogenous level of expression that miR-16 and related family members are involved in the fine-tuning of ERK1 expression (including ERK1/2 phosphorylation).

To test whether the candidate miRNAs target directly the ERK1 mRNA, we performed luciferase assays using the isolated ERK1 3′-UTR. We found that pre-miR-15a, miR-16, miR-195 and miR-497 affected indeed significantly (~70–80%) luciferase expression (Fig. 3D and E). In contrast, a scrambled miRNA sequence showed no effect. We mutated the two potential miR-15 family target sites of the mouse ERK1 3′-UTR (Fig. 3D, see also TargetsScan.org) which abolished partially or completely the suppressing effects of miR-15 family members (Fig. 3E). Next, we could demonstrate that ERK1 mRNA levels were reduced in pre-miR-15a and 195-treated Neuro2a cells, indicating that these miRNAs promote ERK1 mRNA decay (Fig. 3F). These results are consistent with the hypothesis that miRNAs modulate ERK1 mRNA stability in the mouse brain (Fig. 2D). Finally, and consistent with previous findings (23,24), we could show that ERK1/2 and miR-195 are expressed in the same cells in the brain (Fig. 3G).

**The miR-15/ERK pathway can regulate tau phosphorylation**

We next sought independent validation for the role of ERK1/2 in the regulation of tau phosphorylation. For this, we used the inhibitor U0126, which inhibits kinases MEK1/2, ERK1/2 activating kinases. Treatment of Neuro2a cells with U0126 caused a marked decrease in endogenous tau AT8 immunoreactivity, whereas Tau1 reactivity was increased (Fig. 4A). Total tau was not significantly changed in these conditions. Thus, endogenous ERK1/2 activity regulates tau phosphorylation, at least in these cells. We also evaluated the role of the identified miRNAs in regulating tau phosphorylation in primary neurons. Ectopic expression of pre-miR-16 in primary cortical rat neurons induced a marked upregulation of Tau1 stainings (Fig. 4B). Similar results were obtained using pre-miR-15a (data not shown). Notably, no change in total tau was observed in these conditions. We also measured...
endogenous tau phosphorylation in miRNA loss-of-function paradigms. Inhibition of endogenous miR-16 in primary neurons resulted in a significant increase in tau AT8 immunoreactivity (Fig. 4C). We confirmed these changes by western blot (Fig. 4D). Taken together, these findings indicate that specific miRNAs can regulate tau phosphorylation under physiological conditions.

Finally, we explored the hypothesis that loss of miRNA function could contribute to tau pathology in humans. To this end, we performed miRNA qRT-PCR from control and sporadic AD cases. Previous studies have shown that miR-16 is stably expressed in human tissues, including AD brain (25–27). Using this miRNA as internal control, we observed a significant decrease in miR-15a expression levels.
Figure 4. ERK1/2 and miRNA regulation of tau phosphorylation. (A) Western blot analysis of endogenous tau (AT8, Tau1 and total Tau) in the presence of 10 nM U0126 or vehicle (DMSO). Note the isoform-dependent effects (depicted as ‘a’, ‘b’ or ‘c’) on tau phosphorylation (AT8) and dephosphorylation (Tau1) in Neuro2a cells. Triplicate samples are shown. (B) Immunohistochemical studies of endogenous Tau1 (in green) and total tau (in red) in DIV9 rat primary cortical neurons treated with 200 nM of pre-miR-16 or control (pre-miR-1). (C) Immunohistochemistry of endogenous tau AT8 (in green) and total tau (in red) in neurons treated with 200 nM of anti-miR-16 or control (anti-miR-1). In both cases, the nuclei were stained using DAPI. Scale bar = 10 μm (all panels). (D) Anti-miR-16-treated (200 nM) primary neurons were processed for immunoblot analysis using tau AT8 and total tau (Cterm) antibodies. Here, non-transfected (mock) or control (anti-miR-1) mice were used as negative controls. Note the significant (~75%) increase in tau AT8/total tau immunoreactivity in the antisense-treated cells (n = 3 from each group). (E) qRT-PCR of mature miR-15a, miR-15b, miR-195 and let-7a in controls (n = 8–10) and AD patients (n = 8). Relative expression is shown as percentage using the mean of the controls group as reference. The graph mean is shown. In all experiments, ubiquitously expressed miR-16 was used as normalization control. Every data point reflects the mean of three independent RT reactions. Statistical significance was determined by a Student’s paired t-test; *P < 0.05 and **P < 0.001.
in AD brain when compared with healthy controls (Fig. 4C). There was no significant change in the expression of other miR-15 family members, including miR-15b and miR-195. We used as additional control the ubiquitously expressed let-7a, which is not affected in both groups. These results, together with previously published miRNA microarray data (27,28), strongly suggest that specific miR-15 family members, such as miR-15a, are affected in compromised brain displaying tau hyperphosphorylation.

**DISCUSSION**

We show here that loss of Dicer in the adult forebrain impairs the expression of several miRNAs ultimately leading to pathological hyperphosphorylation of tau, the latter being observed in various neurodegenerative disorders. Abnormal tau phosphorylation coincides with neurodegeneration and behavioral abnormalities. We further identify ERK1 (and to a lesser extent ERK2) to be increased in the Dicer knockout mice and provide in vitro evidence that ERK1 could be one candidate effector of tau hyperphosphorylation. We show that miR-15 family members, whose levels are reduced in the Dicer-deficient animals, can regulate ERK1 expression and phosphorylation as well as tau phosphorylation in cultured mouse neurons. Finally, we could show that miR-15a is down-regulated in AD brain. Overall, these results provide strong evidence that misregulation of the miR-15 network may contribute to tau pathology in compromised brain. The biological relevance of our observations is further corroborated by in situ hybridization and immunohistochemistry experiments showing co-expression of miR-195 and ERK1/2 in brain. Obviously, we cannot exclude the contribution of other miRNAs or enzymes in the effects we observed in the Dicer KO mice at this stage of investigation.

In contrast to previous studies (4–7), ablation of Dicer function in mature excitatory neurons results in a mixed degenerative phenotype, characterized by neuronal loss mainly in the CA3/4 areas of the hippocampus and neuronal atrophy which seems predominant in the cortex, which was associated with gliosis. The most salient feature of our model is, however, the dramatic increase in tau phosphorylation, which adds an important component to the neurodegenerative phenotypes observed in various Dicer knockout models. It is noteworthy to mention that abnormal tau phosphorylation and aggregation can cause caspase-3-independent neuronal death in vivo. In addition, sustained activation of ERK can result in neuronal death (29,30). Whereas GFAP and Iba1 stainings were negative in the cortex of 9-week-old mice, we cannot exclude the role of inflammation in mediating tau hyperphosphorylation and neurodegeneration. This possibility warrants further investigation.

It is likely that cell-type or tissue-specific changes in miRNA expression patterns or survival pathways could account for the observed distinctive neurodegenerative phenotypes in different Dicer KO models. We believe that in our model the tau phosphorylation which occurred concomitantly in the different affected brain regions, might explain, at least in part, the observed morphological and behavioral phenotypes. For instance, it is possible that abnormal tau phosphorylation has a direct impact on the cytoskeleton causing neurons to ‘shrink’ in the cortex. In the hippocampus, neurons may be more sensitive to such events resulting in faster neuronal loss. Although toxic accumulation of proteins into autophagic-like vacuoles is another interesting alternative to the cause of neuronal death in the absence of Dicer (4), our preliminary western blot data suggest no changes in autophagic markers LC3-I/II, Beclin1, ATG5-12 and ATG16L1 in the mutant Dicer cKO brain (data not shown). On the other hand, our study strongly suggests that ERK1/2 and tau hyperphosphorylation could contribute to neurodegeneration, but further investigation is needed to address the detailed molecular mechanisms involved in Dicer-mediated neuronal loss in adult neurons.

It is of interest that total tau protein and mRNA levels remain unchanged in Dicer-less brain, which suggests that miRNAs play only a limited role in the direct regulation of tau expression in vivo, at least in the neurons and developmental stage we investigated here. We find, however, that ERK1, one of the kinases phosphorylating tau, was increased at the mRNA, protein and phosphorylation levels in the conditional Dicer KO mice. The marked elevation in ERK1 phosphorylation (∼450%) compared with its protein (∼50%) levels may represent a feed-forward autophosphorylation mechanism of ERK1 (31) and maybe ERK2. The hyperphosphorylation of tau at several sites (Thr181, Ser202, Thr205, Thr231, Ser396, Ser404 and Ser422) is fully consistent with a predominant role of ERK1 (30,32,33). Taken together, these results strengthen the role of ERK1/2 in the pathological phosphorylation of tau in vivo. Whether sustained activation of ERK1 (and ERK2) in the brain can completely recapitulate tau pathology and neurodegeneration, remains an interesting possibility, but difficult to explore in our model given its early lethality.

Our results, combined with others, start to provide quite impressive support for the concept that changes in the miRNA network can contribute importantly to several aspects of the neurodegenerative process in AD and other diseases. This is corroborated by several studies that have detected changes in miRNA expression patterns in AD brain (8,27,34–36). Several of those affected miRNAs seem to participate directly in the regulation of expression of disease-related proteins, namely APP (37–39) and BACE1/β-secretase (27,36), both involved in neurotoxic Aβ peptide production. In this study, we provide evidence that also the other limb of the neurodegenerative process in AD, i.e. the progressive abnormal phosphorylation of tau and potentially its accumulation in tangles, could be promoted by loss of specific miRNAs, which regulate the ERK kinase in AD brain.

In conclusion, our findings add to the accumulating evidence that misregulation of miRNA pathways could contribute significantly to neurodegenerative disorders in humans (8,40–42). One can expect a progressive dysregulation of this finely tuned network with aging, which according to these data would set the stage for various neurodegenerative processes.
MATERIALS AND METHODS

Cells, transfections and treatments

Neuro2A cells were cultured in DMEM/F12 medium supplemented with 10% FCS. Subconfluent cells were transfected with 100 nM of pre-miRs (Pre-miR miRNA Precursor Molecules, Ambion) or anti-miRs (LNA-inhibitors, Exiqon) using LipofectAMINE 2000 following the manufacturer’s instructions. Forty-eight hours post-transfection, cells were processed for immunoblot analysis. For the MEK1/2 inhibition, Neuro2a cells were treated for 48 h with 10 μM U0126 (Sigma) or vehicle (DMSO) prior to immunoblot analysis.

Rat primary cortical cultures were prepared as described previously (43). Nine days after seeding, the culture media was removed and replaced with 500 μl of fresh neurobasal medium supplemented with B27 and glutamine. Cells were incubated 24 h prior to transfection. Then, 1 μl of LipofectAMINE 2000 was diluted in 50 μl of OptiMEM per well (24 wells plate) and incubated at room temperature for 5 min. Fifty microliters of OptiMEM were combined with 2.4 μl solution of 50 μM miRNA (final dilution 200 nM). The diluted miRNA and LipofectAMINE were mixed and incubated at room temperature for 20 min. One hundred microliters of OptiMEM containing the miRNA/LipofectAMINE transfection complexes were added to each well. Tau phosphorylation was evaluated 48 h from the start of transfection.

For the luciferase assays, 100 nM pre-miRs (Ambion) were co-transfected with the full-MAPK3 length mouse 3′-UTR luciferase vector (GeneCopoeia cat# MmiT030696-MT0). Mutagenesis was performed by TopGeneTech (Montreal, Canada) and confirmed by sequencing. Twenty-six to 28 h post-transfection, the measurements were performed using the Dual luciferase reporter assay kit (Promega).

Mice

The generation and characterization of α-CamKII-Cre Tg transgenic mice was described previously (15). The Dicer floxed mice (Dicerlox/lox) were generously provided by M. McManus (University of California, CA, USA) (44). To obtain forebrain-specific Dicer cKO mice (CamKII-Cre/+; Dicerlox/lox), we crossed floxed Dicer (Dicerlox/lox, Dicerlox/fox) mice with CamKII-Cre Tg mice. Experimental mice (CamKII-Cre/+;Dicerlox/lox, designated ‘cKO’) and littermate control mice (CamKII-Cre/+;Dicerlox/lox or +/+; Dicerlox/fox, designated ‘Control’) were used. No differences at the behavioral, morphological and biochemical levels were observed among control mice. All mice were bred on a C57BL/6 background.

Antibodies

For a list of antibodies, please refer to Supplementary Material.

Protein extraction and western blot analysis

Cells were rinsed with cold PBS and lysed in buffer: 1% Triton X-100, 50 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA and complete protease inhibitors (Roche). Protein from mouse brain was extracted using the miRVana PARIS kit (Ambion). Immunoblot analysis was performed as described (45). 2D gel electrophoresis was performed as described previously (46).

RNA extraction and qRT-PCR

Total RNA was extracted from brains and cells using the miRVana PARIS kit (Ambion) according to the manufacturer’s instructions. RT-PCR and quantitative PCR procedures were carried out as described (45). Primer sequences to quantify mouse ERK1 are: forward 5′-ATGAGGCGCCGAAACTACCT-3′, reverse 5′-CCTTACTGTGATGCGCTTG-3′. For miRNA quantifications, probe-specific TaqMan miRNA assays (Applied Biosystems) were used according to the manufacturer’s instructions. Relative expression was calculated by using the comparative C_t method. For mouse quantifications, RNU19 (Applied Biosystems) was used as a normalization control.

Microarray analysis

Microarray details can be found in Supplementary Material.

Immunohistochemistry and in situ hybridization

NeuN, Iba-1 and GFAP immunohistochemical as well as cresyl violet stainings were done as described previously (47). ERK1/2 immunohistochemistry was performed as described previously (48). In situ hybridization for mir-195 was performed as described previously (23, 27). A scrambled miRNA as well as no probe were used as negative controls (data not shown). All assays were done on adult (~2 months old) mice. For primary neurons, cells were fixed in cold 4% paraformaldehyde and 0.05% glutaraldehyde for 30 min at room temperature. Permeabilization was carried out in 0.2% Triton X-100 in phosphate-buffered saline for 10 min. After a 30 min saturation in 2% bovine serum albumin, immunostainings were carried out using AT8, Tau1 and tau total (Cterm) antibodies. Tau1 and AT8 stainings were revealed with a goat anti-mouse IgG (H + L) antibody coupled to Alexa Fluor488 (Molecular Probes). Total tau staining was revealed with a goat anti-rabbit IgG (H + L) antibody coupled to Alexa Fluor568 (Molecular Probes). DAPI was added to the mounting medium. Slides were analyzed with a Zeiss fluorescence light microscope.

Statistical analyses

ERK1/2 and NeuN densitometric quantifications were performed using the ImageJ software. Statistical significance was determined using a Student’s paired t-test as indicated in the text. Calculations were made using the GraphPad Prism 4 software. NeuN positive cells (nuclei) were counted in the text. Calculations were made using the GraphPad Prism 4 software. NeuN positive cells (nuclei) were counted in the text. Calculations were made using the GraphPad Prism 4 software.
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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REFERENCES


