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Dicer Ablation in Oligodendrocytes Provokes Neuronal Impairment in Mice

Daesung Shin, PhD¹, Ji-Yeon Shin, PhD¹, Michael T. McManus, PhD², Louis J. Ptáček, MD^{1,3}, and Ying-Hui Fu, PhD¹

¹Department of Neurology, University of California San Francisco, San Francisco, CA

²Department of Microbiology and Immunology, University of California San Francisco, San Francisco, CA

³Howard Hughes Medical Institute, University of California San Francisco, San Francisco, CA

Abstract

Objective—MicroRNAs (miRNAs) regulate gene expression and have many roles in the brain, but a role in oligodendrocyte (OL) function has not been demonstrated.

Methods—A *Dicer* floxed conditional allele was crossed with the proteolipid protein promoter-driven inducible *Cre* allele to generate inducible, OL-specific *Dicer*-floxed mice.

Results—OL-specific *Dicer* mutants show demyelination, oxidative damage, inflammatory astrogliosis and microgliosis in the brain, and eventually neuronal degeneration and shorter lifespan. miR-219 and its target ELOVL7 (elongation of very long chain fatty acids protein 7) were identified as the main molecular components that are involved in the development of the phenotype in these mice. Overexpressing ELOVL7 results in lipid accumulation, which is suppressed by miR-219 co-overexpression. In *Dicer* mutant brain, excess lipids accumulate in myelin-rich brain regions, and the peroxisomal β -oxidation activity is dramatically reduced.

Interpretation—Postnatal *Dicer* ablation in mature OLs results in inflammatory neuronal degeneration through increased demyelination, lipid accumulation, and peroxisomal and oxidative damage, and therefore indicates that miRNAs play an essential role in the maintenance of lipids and redox homeostasis in mature OLs that are necessary for supporting axonal integrity as well as the formation of compact myelin.

Dicer is essential for generation of functional micro-RNAs (miRNAs), and *Dicer* knockout is embryonic lethal at E7.5.¹ Using a floxed conditional *Dicer* allele crossed with various tissue-specific *Cre* alleles, *Dicer*-mediated miRNAs have been demonstrated to regulate the development of skin progenitors, immune cells, limb outgrowth, chondrocytes, lung, retina, and various neurons.^{2–8} It has been estimated that 70% of miRNAs are expressed in the brain,^{9,10} but little is known about the functions of these brain-specific/enriched miRNAs. Oligodendrocytes (OLs) are glial cells of the central nervous system (CNS) that synthesize myelin, the multilamellar membrane ensheathing axons. Myelin is required for the saltatory conduction of neuronal action potentials and for the maintenance of axonal integrity. Myelin also increases electrical resistance across the cell membrane to prevent the electrical current

from leaving the axon.¹¹ Therefore, damaged myelin impairs the conduction of signals in the affected nerves, causing impairment in sensation, movement, cognition, or other functions depending on which nerves are involved. Demyelinating diseases are caused by genetics, infectious agents, autoimmune reactions, and unknown factors.^{11,12} Recently, miR-23 was reported to regulate the expression of Lamin B1,¹³ which, when duplicated, results in severe myelin loss in autosomal dominant leukodystrophy brain.¹⁴ Overexpression of miR-206 results in downregulation of OL-enriched tubulin polymerization-promoting protein, TPPP/p25, thereby inhibiting OL differentiation.¹⁵ However, the in vivo function of miRNAs in OLs is not known.

To characterize mature OL-specific miRNA functions, we crossed a *Dicer* floxed conditional allele (*Dicer*-floxed)⁴ and proteolipid protein (PLP) promoter-driven inducible *Cre* allele (PLP-CreER^T)¹⁶ to generate inducible OL-specific *Dicer*-floxed mice (hereafter referred to as *Dicer* mutant mice). The *Cre* gene in PLP-CreER^T is a fusion with ER^T and is only activated by Tamoxifen injection. Postnatal OL-specific Cre-loxP recombination deleting the *Dicer* allele dysregulates redox and lipid metabolism of OLs and triggers neurodegeneration (demyelination and inflammatory gliosis). Our results suggest that miRNAs are essential for the maintenance of OLs, likely due to dysregulation of redox and lipid homeostasis.

Materials and Methods

Mice

All experiments with mice were conducted according to protocols approved by the Institutional Animal Care and Use Committee at University of California San Francisco. Tamoxifen-inducible PLP-Cre transgenic mice¹⁶ were purchased from The Jackson Laboratory (Bar Harbor, ME; strain name: B6.Cg-Tg[Plp1-cre/ESR1]3.16Pop/J; stock number: 005975). Floxed *Dicer* mice (strain name: *Dicer*^{flox}) have been described previously.⁴ For CreER^T-mediated recombination, a 10mg/ml Tamoxifen (Sigma, St Louis, MO) solution was prepared in corn oil (Sigma). Two-week-old PLP-CreER^T *Dicer*-floxed mice were injected intraperitoneally for 2 consecutive days, followed by 1 day off, then 2 more consecutive days (total 4×) with 100µg/g body weight.

Immunohistochemistry and Terminal dUTP End-Labeling Stain

Cryosections were prepared from 4% paraformaldehyde-perfused mouse brain, permeabilized with Triton X-100, then blocked and incubated overnight with primary antibodies. After washing with phosphate-buffered saline (PBS), sections were incubated with secondary Cy2- or Cy3-labeled anti-mouse or rabbit immunoglobulin Gs (Amersham, Arlington Heights, IL). After washing (3× for 5 minutes) with PBS, coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA) mounting medium and 4',6-diamidino-2-phenylindole. Primary antibodies used were Cre, *Dicer*, and PLP (Abcam, Cambridge, MA), CC1 (Calbiochem, San Diego, CA), myelin-associated glycoprotein (MAG) (Zymed Laboratories, San Francisco, CA), neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), amyloid precursor protein (APP), and myelin basic protein (MBP) (Chemicon International, Temecula, CA), myelin-oligodendrocyte glycoprotein (MOG) (Santa Cruz Biotechnology, Santa Cruz, CA), F4/80 (AbD Serotec, Oxford, UK), toll-like receptor 2 (TLR2) (eBioscience, San Diego, CA), and fluoro-myelin (Molecular Probes, Eugene, OR). Detection of apoptotic cells with the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) was used according to the manufacturer's directions.

Two-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Two-Dimensional (2D) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) analyses were conducted by Genomine Inc. (Pohang, South Korea). Briefly, whole

mouse brains were homogenized in a lysis solution of 7M urea, 2M thiourea containing 4% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid (CHAPS), 1% dithiothreitol (DTT), 2% Pharmalyte (GE Healthcare, Little Chalfont, UK), and 1mM benzamidine. After centrifugation at 15,000g (1 hour at 15°C), the soluble fraction was used for 2D SDS PAGE. Immobilized pH gradient dry strips (pH4~10NL, 24cm) (Genomine Inc.) were equilibrated for 12~16 hours with 7M urea, 2M thiourea containing 2% CHAPS, 1% DTT, and 1% Pharmalyte, respectively and loaded with 200µg of sample. Isoelectric focusing was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham) following the manufacturer's instructions. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50mM Tris-Cl, pH 6.8 containing 6M urea, 2% SDS and 30% glycerol), first with 1% DTT and then with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 × 24cm, 10~16%). Quantitative analysis of digitized images was carried out using the PDQuest 7.0 (BioRad, Hercules, CA) software according to the manufacturer's protocols. Quantification of spots was normalized by total valid spot intensity. Changes were considered significant if expression deviated at least 2-fold versus control.

Matrix-Assisted Laser Desorption/Ionization Time of Flight Analysis

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis for protein identification was conducted by Genomine Inc. Briefly, protein spots were enzymatically digested in-gel using porcine trypsin. Gel pieces were washed with 50% acetonitrile to remove SDS, salt, and stain; dried to remove solvent; and then rehydrated with trypsin (8~10ng/µl) at 37°C for 8~10 hours. The proteolytic reaction was terminated by addition of 5µl 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration, the peptide mixture was desalted using C₁₈ZipTips (Millipore, Billerica, MA), and peptides were eluted in 1~5µl acetonitrile. An aliquot of this solution was then mixed with an equal volume of a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1µl of mixture was spotted onto a target plate. Protein analyses were performed using an Ettan MALDI-TOF (Amersham).

miRNA Profiling Microarray, Quantitative miRNA Reverse Transcriptase Polymerase Chain Reaction, and Northern Blots

Total RNAs were extracted from mouse brain using miRNeasy mini kit (Qiagen, Valencia, CA). miRNA microarray was conducted by Exiqon (Vedbaek, Denmark). The samples were labeled using the miRCURY Hy3/Hy5 power labeling kit and hybridized on the miRCURY LNA Array (version. 10.0). Quantitative miRNA reverse transcriptase polymerase chain reaction (RT-PCR) was done using a QuantiMir RT Kit (SBI System Biosciences, Mountain View, CA) following the manufacturer's protocol. For miRNA Northern blots, 15µg of total brain RNA was run on 10% urea-acrylamide gels and transferred to N+ nitrocellulose membranes. Digoxigenin (DIG)-labeled miR-219 and U6 (Exiqon) were hybridized at 42°C overnight and detected using the DIG Nucleic Acid Detection Kit (Roche Applied Science, Indianapolis, IN).

Western Blot Analyses

After homogenizing whole brain or myelin fractions in RIPA buffer containing protease inhibitors (Roche Applied Science), total protein extracts were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Milli-pore), and blocked with 5% skim milk or bovine serum albumin (BSA) in Tris-buffered saline/Tween20. Primary antibodies used were glyceraldehyde phosphate dehydrogenase (Chemicon), catalase (Rockland, Gilbertsville, PA), PRDX5 (BD Biosciences, San Jose, CA), GFAP, CD11b/c, cyclic

nucleotide phosphodiesterase (CNPase), and acyl-coenzyme A oxidase 1 (ACOX1) (all from Abcam), PLP, MBP, MOG, and elongation of very long chain fatty acids protein 7 (ELOVL7) (Santa Cruz Biotechnology), MAG (Zymed), and ectonucleotide pyrophosphatase/phosphodiesterase protein 6 (ENPP6) (Novus Biologicals, Littleton, CO).

Reactive Oxygen Species and Eicosanoid Analyses

Peroxide was quantified using the QuantiChrom Peroxide Assay kit (BioAssay Systems, Hayward, CA). Briefly, whole mouse brain was homogenized in ice-cold MSE medium (225mM mannitol, 75mM sucrose, 1mM ethylene glycol tetraacetic acid [EGTA], 5mM HEPES, and 1mg/ml essentially fatty acid-free BSA, pH 7.4).¹⁷ After centrifugation (2× at 12,000g), soluble fractions were quantified using a BCA assay (Bio-Rad Laboratories, Hercules, CA) and used for peroxide quantification according to the manufacturer's manual. For the measurements of eicosanoid levels, we used prostaglandin E₂, thromboxane B₂, and leukotriene B₄ EIA kits (Cayman Chemical, Ann Arbor, MI) per the manufacturer's instructions.

Fast Retrograde Axonal Transport Assay

Mice 3–6 months old were used for retrograde axonal transport assays conducted as previously published.¹⁸

Luciferase Reporter Assay

The constructs in pGL3 promoter and pSV-β-galactosidase control vectors (Promega) were cotransfected into COS-7 cells. All cells were harvested in the reporter lysis buffer (Promega) 48–72 hours post-transfection. Luciferase activity was measured using the Luciferase assay reagent (Promega). The β-galactosidase activities in the reporter lysis buffer were measured with a spectrophotometer using the β-galactosidase enzyme assay system (Promega) and normalized to luciferase.

Plasmid Construction and Site-Directed Mutagenesis

Three-prime untranslated regions (3'UTRs) and coding regions plus 3'UTRs of ELOVL7 and ENPP6 were amplified by RT-PCR from mouse brain total RNA. 3'UTR regions were subcloned into the *Xba*I site of pGL3 promoter vector (Promega). Coding regions with 3'UTRs were ligated into pCMVTag2B (Stratagene, San Diego, CA). mmu-miR-32, mmu-miR-144, and mmu-miR-219 were subcloned into pIRES2-DsRed2 (Clontech, Mountain View, CA). Quik-change mutagenesis kit (Stratagene) was used for site-directed mutagenesis. All sequence information on the primers used in this study is available on request.

In Situ Hybridization

mmu-miR-219 in situ hybridization was performed as described previously¹⁹ with DIG-labeled probe (Exiqon).

Clinical Behavior Analysis

Mouse phenotypes were recorded monthly using a standard protocol,²⁰ except that stage III was subdivided to III–1 (severe hind limb ataxia only) and III–2 (severe hind limb ataxia with kyphosis).

Luminometric Assay for Peroxisomal β-Oxidation

Peroxisomal β-oxidation activity was measured as previously reported,²¹ using the activity of acyl-CoA oxidase, the rate-limiting enzyme for peroxisomal β-oxidation. Briefly, mouse brain homogenates (10%, weight/vol) were prepared in media containing 300mM mannitol, 0.1mM

EGTA, and 10mM HEPES, pH 7.2, and centrifuged for 1 minute at 1,000g. Resulting supernatants were used for all enzyme assays. The assay mixture contained 100mM potassium phosphate, 4mM NaN₃, 10μg microperoxidase/ml, 20μM luminol, 0.5mg of defatted BSA/ml, 0.1mM oxidized form of nicotinamide adenine dinucleotide, and 5mM ethylenediaminetetraacetic acid, pH 8.5. The reaction was started by the addition of 25μM palmitoyl-CoA. Controls not containing protein (≥ 3) were included with each assay. Net luminescence signal was taken as sample signal after subtraction of the corresponding blank value.

Adipocyte Differentiation and Oil Red-O Stain

The constructs used in Figure 5E were cotransfected with miR-219 in preadipocytes (3T3-L1), and the cells were differentiated to mature form using the Adipogenesis Assay kit (Cayman Chemical). Preadipocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% calf serum to confluence. Two days postconfluence, the cells were differentiated for 3 days using DMEM containing 10% fetal bovine serum (FBS), 1μM dexamethasone, 0.5mM isobutylmethylxanthine, and 5μg/ml insulin. After 2 days, the cells were switched to DMEM containing 10% FBS and 5μg/ml insulin and further incubated for 4 days. For Oil Red O staining, differentiated cells were fixed for 15 minutes with solution from the kit. After 3 washes, fixed cells were stained with Oil Red-O solution for 20 minutes and washed with water and wash solution before collection of microscopy images. Stained cells were dried completely and treated with dye extraction solution for 30 minutes with shaking prior to quantitating lipid by optical density of extracts at 492nm.

Results

OL-Specific Conditional *Dicer* Knockout Mouse Model

To investigate the function of *Dicer* in OLs, we generated inducible PLP-CreER^T conditional *Dicer* knockout mice (PLP-CreER^T; *Dicer*^{flox/flox})⁴ by crossing *Dicer*^{flox} with the PLP-CreER^T conditional mouse.¹⁶ CreER^T is a fusion of the Cre recombinase and a mutated ligand binding domain of the human estrogen receptor and is activated only by Tamoxifen. Here, CreER^T expression is under the control of PLP promoter and therefore allows us to obtain both inducible and site-specific recombination (Fig 1A). To ensure that Cre protein is specifically expressed in OLs, we costained Cre antibody with major neuronal cell markers such as NeuN (neurons), GFAP (astrocytes), PLP (membrane of OLs), and CC1 (OL soma). As expected, Cre protein is coexpressed exclusively with PLP and CC1 in the major myelin-enriched regions such as the corpus callosum and cerebellum, indicating that Cre expression is confined to OLs in this mouse model (Supplementary Fig 1A).

OL-Specific *Dicer* Deletion Causes a CNS Phenotype

Without Cre activation, *Dicer* mutant mice are indistinguishable from wild-type littermates (data not shown). Induction of Cre-loxP recombination was achieved by Tamoxifen injection (4×, see Fig 1). Although PLP-CreER^T-dependent *Dicer* deletion is restricted to mature OLs, less expression of *Dicer* is discernible via Western blot analysis of whole brain extracts and immunohistochemistry (see Supplementary Fig 1B) of *Dicer* mutant mice. On PLP-CreER^T-dependent removal of *Dicer* in OLs, we surprisingly observed signs of CNS impairment (ataxia, paralysis, kyphosis, and early death) that were similar to those in mice having OL-specific peroxisome ablation²⁰ and CNPase knockout.²² Therefore, based on previously established clinical classification, we monitored the phenotypes monthly and found that the mice start to show hind limb ataxia at ~2 months of age that becomes severe by 3~4 months of age. Mice deteriorate progressively until they develop paralysis and kyphosis. Approximately 30% of *Dicer* mutant mice died before 6 months, and 90% died within 1 year. Ten percent of mutant mice survived >1year. This life-span variation probably comes from inconsistency of the

penetrance of PLP-CreER^T allele in each individual, although the induction of Cre-mediated recombination was found primarily in major myelin-enriched brain regions (see Supplementary Fig 1C). OL-specific *Dicer* null mice also showed weight loss and motor-coordination defects. Mutant mice showed no significant abnormalities in gross brain morphology.

***Dicer* Mutant Mice Show Reduced Myelin Proteins**

Because the mice were designed for specific knockout in mature OLs, we checked the expression of myelin proteins using a broad panel of myelin antibodies. At 1 month of age, myelin protein levels were indistinguishable between wild-type and mutant mice, but all were reduced in older *Dicer* mutant mice (6 months) compared with wild-type littermates (Fig 2A). Immunohistochemical investigation of myelin using fluoro-myelin and PLP antibody showed that *Dicer* null mice have less myelin in corpus callosum and cerebellar white matter tracts (see Fig 2B). These data suggest that post-natal OL-specific *Dicer* deletion causes demyelination. Because apoptosis of OLs has been suggested to partially account for the demyelination in some myelin disorders,²³ terminal dUTP end-labeling (TUNEL) assays were performed in *Dicer* mutant and wild-type mice. Wild-type brain showed no discernible apoptosis, but conditional *Dicer* deleted brain showed dramatically increased apoptosis in cells of cerebellum and spinal cord of adult animals, indicating that apoptotic cell death may account, at least in part, for demyelination (see Fig 2C).

Inflammatory Gliosis Is Upregulated in *Dicer* Mutant Mice

To determine whether the OL-specific *Dicer* deletion affects other glial cells, we analyzed astrocytes and microglia with specific markers such as GFAP (astrocytes) and CD11b/c (microglia). Surprisingly, Western blot analyses revealed that GFAP level is approximately 3.5-fold, and CD11b/c is about 2-fold higher in the mutant brain (see Fig 2). Histological analysis indicated that the GFAP signal is very strong in the *Dicer* mutant, with the most extensive astrogliosis in corpus callosum, cerebellum, and brainstem. Staining of the microglia surface molecule F4/80 is significantly higher in *Dicer* null mice. TLR2 is only expressed in activated microglia,²⁴ and was increased in corpus callosum, cerebellum, midbrain, and spinal cord from mutant mice but not as significantly in cerebral cortex and hippocampus. We analyzed the production of eicosanoids (prostaglandins, thromboxanes, and leukotrienes), which are byproducts of demyelination and can trigger inflammation by attracting lymphocytes.¹² Using an enzyme immunoassay in brain extracts, we found significantly higher levels of eicosanoids in mutant versus control. Thus, *Dicer* mutant mice show not only demyelination, but also evidence of inflammatory gliosis.

Axonal Degeneration Is Triggered in *Dicer* Mutant Mice

Because myelin plays a critical role in axon function, nerve conduction can be impaired or lost in *Dicer* null mice. The presence of motor coordination defects in mutant mice indicates an underlying neuronal impairment. To test whether this impairment might in part be due to altered axonal transport, we stained wild-type and mutant brain for APP. APP can be used to show axonal transport defects because it accumulates within the trajectory of degenerative axons in cases where axonal transport is disturbed.²⁵ APP staining was increased in mutant brain compared with control (Fig 3A). We directly measured retrograde axonal transport by injecting fluorescein-conjugated cholera toxin B subunit into the right superior colliculus and assayed fluorescent cells 18 hours later in the retina. Mutant mice showed significantly fewer labeled cells compared with wild-type mice, suggesting axonal degeneration in OL-specific *Dicer* knockout (see Fig 3B). Future detailed molecular and morphometric characterizations will further clarify whether the axonal degeneration observed in these mice was due to loss of axons or other secondary axonal degeneration.

Increased Oxidative Stress in *Dicer* Mutant Mice

Because *Dicer* regulates the generation of various miRNAs, and a single miRNA can modulate a large number of target proteins, there may be many factors contributing to the phenotypes of OL-specific *Dicer* knockout. To identify target proteins that contribute to the mouse phenotype, we performed 2D SDS-PAGE analysis in search of altered protein expression in *Dicer*-deficient OLs. Because the primary function of miRNAs is the repression of target proteins at the post-transcriptional level, we assumed that suppression of *Dicer*-mediated miRNA generation would trigger increased expression of direct protein targets responsible for the phenotype. Total protein extracts of whole brains from 2 pairs of 2-month-old *Dicer* mutant mice and wild-type littermates were subjected to 2D SDS-PAGE analysis. Comparison of the relative densities of ~2000 detectable protein spots on each gel identified 16 proteins with >2× higher abundance in mutant as opposed to control mice (Fig 4A and Supplementary Fig 2). They fell into the following functional classes: vesicle transport, stress/redox regulation, and actin-cytoskeleton regulation.

Interestingly, it is known that stress and redox-state regulatory proteins are highly upregulated on oxidative damage like that found in neurodegenerative disorders including Parkinson disease, Alzheimer disease, and amyotrophic lateral sclerosis,^{26,27} although it is unclear whether oxidative stress is a primary trigger or merely a downstream consequences of neurodegeneration. To test whether reactive oxygen species (ROS) are more abundant in the OL-specific *Dicer* deleted brain, we assayed peroxide levels by enzyme-linked immunosorbent assay and found much higher levels in the mutant brains (see Fig 4B). To further verify upregulated oxidative stress, we analyzed the levels of redox regulatory proteins by Western blot. Catalase, which detoxifies peroxides and is upregulated in the setting of oxidative stress,²⁸ is increased in OL-specific *Dicer* deleted mice with severe defects such as paralysis and kyphosis (see Fig 4C, D). Peroxiredoxin, an antioxidant enzyme controlling peroxide levels,²⁹ was highly upregulated in whole brain and in myelin fractions from mutant mice, suggesting that oxidative stress is significantly increased in OL-specific *Dicer* null mouse brain. Thus, OL-specific *Dicer* mutant mice show clinical neurodegeneration and oxidative stress.

OL-Specific Deletion of *Dicer* Reduces the Production of miR-32, miR-144, and miR-219

To investigate whether OL-specific *Dicer* ablation indeed results in the reduction of mature miRNAs in the brain, we conducted miRNA microarray analyses (Supplementary Fig 3). Reduced miRNAs in OLs from older, severely affected mutant mice may be masked by increase miRNA due to proliferation of astrocytes and microglia. Therefore, we used 1-month-old mutant mice (before gliosis is evident) and still found reduced levels of *Dicer* (see Fig 1C). miRNA microarrays revealed that miR-32, miR-144, and miR-219 are downregulated, whereas miR-7a, miR-7b, miR-181a-1, and miR-592 are upregulated in the OL-specific *Dicer* mutant compared with controls. We confirmed upregulation of all of these using quantitative miRNA real-time RT-PCR, which consistently matched the microarray results. Because the primary affect of *Dicer* ablation is to reduce miRNA production, we focused on the 3 downregulated miRNAs as the primary miRNA candidates that may be responsible for the phenotype. Interestingly, miR-219 has been reported as the most abundant miRNA in mature OLs.³⁰ Using in situ hybridization, we found that miR-219 is indeed highly expressed in white matter of cerebellum and corpus callosum, and is greatly reduced in the OL-specific *Dicer* mutant brain (Supplementary Fig 4). We therefore focused on miR-219 as a primary miRNA candidate for causing the mutant phenotype. Because both microarray and quantitative real-time RT-PCR can detect pri- and pre-miRNA molecules and mature forms, it is possible that the reduced miRNAs are masked by premature forms. Northern blotting was thus used to analyze levels of mature miR-219 in mouse brain. Both young (1.5 months) and older (6 months) mice showed miR-219 to be reduced more than 5-fold in *Dicer* mutant mice.

Although miR-219 is known to be enriched in mature OLs, the temporal expression pattern has not been carefully studied. We examined miR-219 expression during mouse development using quantitative real-time RT-PCR and Northern blotting (Supplementary Fig 5). miR-219 becomes detectable beginning at P7, peaks at P21, and then decreases slightly to a plateau after P50. Interestingly, the period for the induction of Cre-loxP recombination in this mouse model (between P14 to P21) occurred right before miR-219 reached peak levels.

ELOVL7 Is the Target of miR-219 and Is Increased in OL-Specific *Dicer* Mutants

To identify immediate downstream proteins regulated by miR-219, we investigated 3'UTR sequences of upregulated proteins identified in 2D SDS-PAGE analysis (Fig 4A) using Targetscan software. However, none of the upregulated proteins was predicted to be a direct target of miR-219. We next examined a public database of highly expressed genes in the mature OLs,³¹ and found that 2 of the OL-enriched genes have strong putative miR-219 binding regions, ELOVL7 and ENPP6. Western blot analysis in brain extracts from OL-specific *Dicer* null mice and wild-type littermates revealed that ELOVL7 levels are greatly increased in 6-month-old mutant mice compared with controls, whereas no difference was observed in 1-month-old mice (Fig 5). Expression of ENPP6 was not changed at either age. We used luciferase reporter assay with the pGL3 promoter vector containing the 3'UTRs of either ELOVL7 or ENPP6 to further test whether ELOVL7 and ENPP6 are indeed regulatory targets of miR-219. Both 3'UTR sequences resulted in reduced luciferase activity in the presence of miR-219 compared with negative vector controls. To further assess whether the suppression of both genes is miR-219 specific, miR-32 or miR-144 was cotransfected into the cells. Interestingly, these miRNAs do not reduce the reporter activities of ELOVL7 3'UTRs, but still reduce the activity of ENPP6 3'UTRs, suggesting that ELOVL7, but not ENPP6, is a specific target of miR-219. Finally, we carried out luciferase and Western blot analyses using ELOVL7 both with and without the putative miR-219 binding site in the 3'UTRs. Deletion of the putative miR-219 binding region relieves the suppression of ELOVL7 expression, showing that ELOVL7 is a primary and specific target of miR-219 via this 3'UTR sequence and is upregulated in OL-specific *Dicer* mutant brain.

ELOVL7 Synthesizes Lipids That Are Accumulated in the *Dicer* Mutant Brain

Seven elongases named ELOVL1-7, have been identified in mammals and are suggested to perform the condensation reaction in the elongation cycle of very long chain of fatty acid (VLCFA) synthesis.^{32,33} To examine the effect of the increased ELOVL7 in vitro, we tested whether fatty acids accumulate in the presence of ELOVL7 and miR-219 in differentiated adipocytes (Fig 6A) and then quantified accumulated lipid droplets by extracting Oil Red-O from stained cells and measuring its optical density (see Fig 6B). There was decreasing lipid accumulation as miR-219 concentrations were increased in cells transfected with ELOVL7 containing the wild-type 3'UTR but not when transfected with ELOVL7 in which the putative 3'UTR miR-219 binding site was deleted, suggesting that ELOVL7 triggers lipid accumulation and miR-219 regulates the function of ELOVL7 through binding to this 3'UTR sequence. To confirm this finding in vivo, we determined whether neutral lipids accumulate at higher levels in *Dicer* mutant brain compared with wild-type. We analyzed the brains of 6-month-old *Dicer* mutant and wild-type littermates using Oil-red O and found that lipid inclusions were most distinct in the *Dicer* null brain (see Fig 6C and Supplementary Fig 6).

Activity of Peroxisomal β -Oxidation Is Downregulated in OL-Specific *Dicer* Mutant Brain

Absence of functional peroxisomes in OLs causes astrogliosis, widespread axonal degeneration, and progressive subcortical demyelination.²⁰ Because the OL-specific *Dicer* mutant phenotype is very similar to that of the OL-specific peroxisome knockout mouse, we searched for any peroxisomal proteins that might be targets of miR-219 using Targetscan.

ACOX1 (palmitoyl) was identified to have a very strong putative miR-219 binding site in the 3'UTR (data not shown). However, ACOX1 is dramatically reduced in *Dicer* mutant brain, as shown in Figure 6D, suggesting that ACOX1 is not a direct target of miR-219. Because ACOX1 is a rate-limiting enzyme of the peroxisomal β -oxidation pathway,³⁴ we surmised that peroxisomal β -oxidation might be impaired in mutant brain. β -Oxidation was measured in whole brain extracts, and the activity of the β -oxidation pathway in mutant brain was reduced to approximately 0.5-fold of that in wild-type littermates (see Fig 6E).

Taken together, our results suggest that PLP promoter-driven *Dicer* deletion after postnatal day 14 suppresses miRNA generation (especially miR-32,-144,and-219) in mature OLs. Protein levels for many downstream targets are increased, including myelin-enriched ELOVL7. Increased ELOVL7 could lead to VLCFA accumulation, which results in demyelination and oxidative damage. In parallel, other unknown pathways could damage the peroxisomal β -oxidation, which also causes VLCFA accumulation and oxidative damage. These pathways together then trigger inflammation and neuronal degeneration, with growth retardation and premature death in *Dicer* mutant mice (see Fig 6F).

Discussion

To explore the role of miRNAs in mature OLs, we used inducible *Dicer* ablation by PLP promoter-driven Cre-loxP recombination. It has previously been shown that the expression of transgenes driven by PLP promoter is primarily restricted to myelinating cells (see Supplementary Fig 1C).³⁵ PLP expression begins during embryonic day 7 and at early developmental stages of the OL lineage.^{35–37} Therefore, PLP promoter-driven CreER^T mice are useful for the study of mature OLs from embryonic stages to adulthood. In this study, we examined the role of miRNAs in myelinating OLs in adulthood because *Dicer* ablation at earlier embryonic stages may cause very pleiotropic affects that could complicate interpretation. Initially, we induced Cre-loxP recombination at 1 month of age (P30), and the mice showed similar but milder symptoms to those shown in Figure 1 after 6 months of age (data not shown). We therefore chose to inject Tamoxifen starting at postnatal day 14. Interestingly, our model triggers inflammatory neurodegeneration and demyelination (see Figs 2–3). Previously, CNPase-Pex5 (OL-specific peroxisome ablation), PLP-deficient, and CNP1-null mice have been reported to have similar axonal degeneration features as well as demyelination.^{20,22,38} Thirty-five percent of myelin is composed of phospholipids, which are degraded by phospholipase A₂ in a reaction that releases arachidonic acid and lysophospholipid. Arachidonic acid can be further metabolized by cyclooxygenases to prostaglandins or thromboxanes, whereas lipoxygenases catalyze the conversion to leukotrienes. These eicosanoids are potent mediators of inflammation as lymphocyte chemoattractants¹² and are increased in neurodegenerative diseases, such as Parkinson disease, Alzheimer disease, multiple sclerosis, and leukodystrophies.³⁹ In this study, PLP promoter-driven Cre-loxP recombination deleted *Dicer* in the restricted OLs, and resulted in pronounced demyelination, with dramatic increase of eicosanoids, suggesting that inflammatory neurodegeneration in these mice might be caused by lipid breakdown products from demyelination.

To our surprise, we found only 7 miRNAs significantly regulated by postnatal OL-specific *Dicer* ablation (see Supplementary Fig 3B, C), and the maximum change was only 2-fold. There are several possible reasons for this result. First, PLP-CreER^T is only expressed in mature OLs, which represent a small subset of total brain cells, although it is also detected with less expression in Schwann cells and even less in the heart and testis.¹⁶ The use of whole brain extracts may have diluted changes in miRNA levels in OLs. Accumulated pools of mature miRNAs from before *Dicer* ablation may not have been totally depleted, due to the use of young (1 month old) mice. Third, it is known that *Dicer* regulates only the maturation of miRNAs. Microarray and quantitative real-time RT-PCRs cannot distinguish pri- and pre-

miRNAs from mature miRNA,⁴⁰ therefore potentially masking reductions further. In agreement with this notion, Northern blot analysis showed that the functionally mature form of miR-219 is much less prevalent (5-fold reduction) in *Dicer* mutant brain (see Supplementary Fig 3D). In addition, the mosaicism existing in inducible systems could also influence the outcome of our studies. miR-219 is the most abundant miRNA in mature OLs,³⁰ but miR-32 and miR-144 were not listed in that study. Therefore, we chose to focus on miR-219 as a candidate miRNA for our *Dicer* mutant phenotype. miR-219 negatively regulates Ca²⁺ influx through N-methyl-D-aspartate (NMDA) receptor signaling transduction in cortical cells and CaMKII γ , a downstream effector of NMDA-mediated Ca²⁺ signaling, is a target of miR-219.⁴¹ miR-219 also modulates the circadian clock located in the suprachiasmatic nucleus via regulation of the CLOCK and BMAL1 complex.⁴² In this study, we show that ELOVL7, which is highly expressed in mature OLs,³¹ is specifically regulated by miR-219, and is significantly increased in our *Dicer* mutant mice.

Synthesis of VLCFAs is carried out in the endoplasmic reticulum, and the first reaction, condensation of fatty acyl-CoA and malonyl-CoA, is the rate-limiting step performed by members of the ELOVL protein family.^{32,33} We show here that ELOVL7 can elongate fatty acids, as ELOVL7 overexpression triggers lipid accumulation in differentiated adipocytes and is suppressed by miR-219 co-overexpression. These results indicate that miR-219 can regulate the synthesis of VLCFA by ELOVL7 in OLs. Future mouse models overexpressing ELOVL7 or suppressing miR-219 will validate these results. Although fatty acids are necessary chemicals in the body, excess amounts must be removed by β -oxidation in both mitochondria and peroxisomes. Accumulation of VLCFA in white matter and adrenal cortex is the principal biochemical abnormality in X-linked adrenoleukodystrophy (X-ALD). VLCFA oxidation takes place only in peroxisomes,⁴³ and ACOX1 is the first and rate-limiting enzyme of the peroxisomal β -oxidation pathway.³⁴ Defective peroxisomal β -oxidation causes the impairment of lipid metabolism and VLCFA accumulation.⁴⁴ Excess VLCFAs incorporate in cell membranes of white matter tracts and may disrupt the stability of membranes by occupying the lateral chains of proteolipid proteins, gangliosides, and phospholipids.^{44,45} Dramatic accumulation of fatty acids in cell membranes results in elevated oxygen consumption, which can trigger oxidative damage,⁴⁶ a common finding in a number of neurodegenerative diseases.²⁶ It is also known that free radical generation is involved in symptomatic X-ALD patients, where reduction in ROS scavenging and/or ROS overproduction is thought to occur,²⁷ and as we now see in *Dicer* mutant mice.

In summary, our data demonstrate that *Dicer* plays a significant role in mature OLs in vivo. Postnatal *Dicer* ablation in OLs causes inflammatory neurodegeneration through increased demyelination, lipid accumulation, and both peroxisomal and oxidative damage. Characterization of the essential roles of miRNAs in the maintenance of lipids and redox homeostasis in mature OLs may provide insights into novel therapeutics for demyelinating, neuroinflammatory disorders such as X-ALD and multiple sclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Postnatal *Dicer* deficiency in oligodendrocytes (OLs) causes neuronal impairment. (A) LoxP-flxed *Dicer* mice were crossed with proteolipid protein (PLP) promoter-driven inducible Cre mice to get Tamoxifen (TM)-inducible OL-specific *Dicer* knockout mice. TM allows nuclear translocation of Cre protein, where it mediates recombination via Cre-loxP. (B) TM was injected intraperitoneally at postnatal days 14, 15, 17, and 18. BW = body weight. (C) Polymerase chain reaction (PCR) of genomic DNA shows the presence of Cre and both *Dicer* wild-type and loxP-flxed alleles. TM-dependent Cre induction causes reduced *Dicer* expression in PLP-Cre– driven *Dicer* floxed mice (1 month old). p Values were calculated using an unpaired Student *t* test. Data are expressed as means $\pm$ standard deviation (SD). WB = Western Blot. (D) Clinical phenotypes were monitored monthly. The mouse in the figure is a 5-month-old TM-injected mutant (*Dicer*^{flx/flx}; PLP-CreERT⁺). (E) Survival curve for mutant mice. (F) *Dicer* mutants show weight loss and incoordination. Data are expressed as means $\pm$ SD. (G) Gross brain morphology shows no obvious differences in TM-injected mutant versus control mice at 5 months of age (mutants rated clinical stage V).

Fig 2.

Demyelination caused by the loss of *Dicer* in oligodendrocytes triggers gliosis and inflammation markers. (A) Myelin protein expression is reduced in adult mutant (MT) brain. (B) Fluoro-myelin and proteolipid protein (PLP) antibody staining on cryosections of mutant brain shows much less compact myelin in the corpus callosum and cerebellum white matter tracts. (C) *Dicer* mutants show prominent terminal dUTP end-labeling (TUNEL) staining in the white matter of cerebellum and spinal cord compared to the wild-type (WT). (D) Western blotting showed dramatic increases in markers of astrocytes (glial fibrillary acidic protein [GFAP]; 3.5 fold) and microglia (CD11b/c; 2 fold) in mutant brain. (E) Astrocytosis was confirmed using immunohistochemistry against GFAP in cerebellum, spinal cord, corpus callosum, and superior colliculus. (F) Microgliosis was confirmed using antibodies against a microglia-specific surface marker (F4/80, top panel) and against toll-like receptor 2 (TLR2), which is only expressed in activated microglia in cerebellum, corpus callosum, midbrain, and spinal cord in mutant versus wild-type. (G) Enzyme immunoassays detecting eicosanoids in whole brain extracts revealed > 1.5-fold higher levels in mutant compared with wild-type. Scale bars represent 100µm in B, C, and E, and 25 µm in F. Data are expressed as means ± standard error of mean. MBP = myelin basic protein; MAG = myelin-associated glycoprotein; CNPase = cyclic nucleotide phosphodiesterase; MOG = myelinoligodendrocyte glycoprotein; GAPDH = glyceraldehyde phosphate dehydrogenase; DAPI = 4',6-diamidino-2-phenylindole.

Fig 3.

Loss of *Dicer* in oligodendrocytes (OLs) leads to axonal degeneration. (A) Detection of amyloid precursor protein (APP) by immunohistochemistry showed that axonal swellings appear in the myelin-enriched brain regions of mutant mice. (B) A fast retrograde axonal transport assay revealed decreased accumulation of fluorescent signal in retinal ganglion cells in mutant versus litter-mate control mice, suggesting that loss of *Dicer* in OLs causes defective axonal transport. Scale bars represent 50 μ m in A and 100 μ m in B. PLP = proteolipid protein; CTB = cholera toxin B subunit; FITC = fluorescein Isothiocyanate.

Fig 4.

Oxidative stress caused by *Dicer* deficiency. (A) Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis using whole brain extracts revealed that the proteins associated with vesicle transport, stress, redox, and actin cytoskeleton regulations are highly increased in the mutants. (B) H_2O_2 is increased in *Dicer* mutant brain. Data are expressed as means $\pm$ standard deviation. $*p < 0.001$. (C) Confirmation of upregulated stress and redox regulatory proteins, catalase and peroxiredoxin, in brains of adult mutant mice with severe phenotypes. (D) Immunohistochemical staining shows upregulated catalase in white matter tracts of cerebellum in mutant mice. Neuronal nuclei (NeuN) staining indicates grey/white matter boundaries in cerebellum. Scale bar represents 100 μ m. PLP = proteolipid protein; WT = wild-type; MT = mutant; GAPDH = glyceraldehyde phosphate dehydrogenase.

Fig 5.

ELOVL7 (elongation of very long chain fatty acids protein 7) is a specific miR-219 target. (A) Western blot analyses using brain extracts from 1- and 6-month-old mutant mice versus control showed that ENPP6 (ectonucleotide pyrophosphatase/phosphodiesterase protein 6) is not upregulated in the mutants and that ELOVL7 is, although only at the 6 month time point. (B) miR-219 suppresses in vitro expression of both ELOVL7 and ENPP6 encoding genes in a dosage-dependent manner. $*p < 0.01$. (C) ELOVL7 expression is suppressed by miR-219, but not by miR-32 and -144. ENPP6 is suppressed by all 3 microRNAs. (D) The miR-219 binding-site deletion partially relieves miR-219-mediated ELOVL7 suppression. $*p < 0.001$ and $**p < 0.005$. (E) The 3-prime untranslated region (3'UTR) and coding sequences of ELOVL7 were cloned into a mammalian expression vector as a FLAG-tag fusion under the cytomegalovirus promoter (pCMV). Either wild-type or miR-219 binding site deleted mutant construct was cotransfected with miR-219 into COS-7 and NIH-3T3 cells. Western blotting against ELOVL7 revealed that miR-219 suppresses ELOVL7 expression, which is recovered by the binding site mutation. Data are expressed as means $\pm$ standard error of mean. WT = wild-type 3'UTR of ELOVL7; MT = miR-219 binding site-deleted 3'UTR. GAPDH = glyceraldehyde phosphate dehydrogenase; PLP = proteolipid protein.

Fig 6.

Oligodendrocytes (OLs)-specific *Dicer* ablation triggers lipid accumulation and peroxisomal β -oxidation malfunction in the brain. (A) ELOVL7 (elongation of very long chain fatty acids protein 7) overexpression resulted in lipid accumulation in the absence of miR-219. Addition of miR-219 triggered a dramatic reduction of lipid droplets in the cells transfected with wild-type (WT) but not mutated 3-prime untranslated region (3'UTR). Scale bar represents 1cm. (B) Quantification of the accumulated lipid in WT and mutant (MT) ELOVL7 constructs in adipocyte cells. The dye was extracted, and absorbance was measured at 492nm. p Values were calculated by an unpaired Student *t* test. Data are expressed as means $\pm$ standard deviation. (C) Brain sections were stained with Oil Red-O and showed much more lipid accumulation in MT than WT littermate mice. Scale bar represents 500 μ m. (D) Western blotting showed reduced acylcoenzyme A oxidase 1 (ACOX1) levels in older mutant mice indicating that ACOX1 is not a direct target of miR-219. (E) An assay for peroxisomal β -oxidation showed less activity in brain extracts from MT mice versus WT. p Values were calculated using an unpaired Student *t* test. Data are expressed as means $\pm$ standard error of mean. (F) A model of downstream effects caused by postnatal OL-specific *Dicer* ablation. Mature OL-specific *Dicer* ablation suppresses the generation of miR-32, miR-144, and miR-219. Myelin-enriched ELOVL7 synthesizes more lipids such as very long chain of fatty acids (VLCFAs), resulting in demyelination and oxidative damage. In parallel, other unknown proteins targeted by these micro-RNAs (miRNAs) may regulate peroxisomal function, also contributing to lipid accumulation, oxidative damage, and ultimately, inflammatory gliosis and neurodegeneration in OL-specific *Dicer* mutant mice. PLP = proteolipid protein; GAPDH = glyceraldehyde phosphate dehydrogenase.