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Age-dependent neuron loss is associated with impaired adult neurogenesis in forebrain neuron-specific Dicer conditional knockout mice

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ABSTRACT

Impairment in the microRNA (miRNA) network causes a number of neurodegenerative diseases. Endoribonuclease Dicer is a key RNase to produce mature miRNAs. It has been shown that Dicer is important for the maintenance of excitatory neuron survival during early postnatal period. However, the role of Dicer in adult mature excitatory neuron survival is not clear. In this study, we generated a mouse model in which Dicer is conditionally inactivated in forebrain excitatory neurons from a mature stage, and this line is termed *Dicer* conditional knockout (cKO). Significant age-dependent neurodegeneration was observed in the cortex of *Dicer* cKO mice, indicating an important role of Dicer in the maintenance of mature excitatory neuron survival in the adult cortex. Impairment in adult neurogenesis was found in 6-month but not in young *Dicer* cKO mice. However, astrocytosis was detected in young *Dicer* cKO mice displaying no apparent neuron loss. Overall, neurogenesis impairment and neuroinflammation may play pivotal roles in the progression of neurodegeneration.

1. Introduction

Neurodegeneration represents a group of chronic diseases pathologically characterized by progressive loss of neurons in distinct areas of the adult brain. Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) belong to this category of disorder (Schon and Przedborski, 2011). Although neurodegenerative patients usually survive more than a decade after the diagnosis of disease, the onset of neurodegeneration may take place many years before clinical symptoms start. Currently, the mechanism underlying progressive neurodegeneration is poorly understood. Moreover, there is no effective treatment or prevention for this disorder (Selkoe, 2012).

It has recently been demonstrated that microRNAs (miRNAs) play critical roles in neurodegenerative diseases (Hébert et al., 2008). Evidence has shown that impairment on the miRNA network causes neurodegeneration (Hébert and De Strooper, 2009; Hébert et al., 2010). A number of mouse models with impaired miRNA network through conditional deletion of Dicer have been generated. One study demonstrated that loss of Dicer in excitatory neurons from an embryonic stage (E15.5) results in early postnatal death in mice (Davis et al., 2008). Conditional inactivation of Dicer in excitatory neurons from postnatal day18 (P18) results in tau hyperphosphorylation, neuron loss and early adulthood death (Hébert et al., 2010). Moreover, it has been reported that conditional deletion of Dicer in dopamine or Purkinje neurons leads to neurodegeneration in midbrain (Pang et al., 2014; Kim et al., 2007) or cerebellum (Schaefer et al., 2007). Dicer or the miRNA network is also important for the developing brain (Kawase-Koga et al., 2009, 2010).

Neurogenesis is defined as a process of generating functional neurons from neural stem cells in the brain of mammals (Gage, 2000). It takes place specifically in two brain areas, the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) around the lateral ventricles (Gage, 2002). Compromised neurogenesis contributes to the progression of neurodegeneration (Lazarov and Marr, 2010). It has been reported that both the pool

Abbreviations: miRNA, microRNA; cKO, conditional knockout; AD, Alzheimer's disease; DG, dentate gyrus; SGZ, subgranular zone; SVZ, subventricular zone; GCL, granular cell layer; MAP2, microtubule-associated protein; SVP38, synaptophysin; GFAP, glial fibrillary acidic protein; NPC, neural progenitor cell; DCX, doublecortin; PSD95, post-synaptic density 95.

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of neural stem cells and their proliferative potential are markedly diminished in AD (Brinton and Wang, 2006). Altered neurogenesis have been observed in the adult brain of various AD mouse models (Rampon et al., 2000; Chen et al., 2008; Rodriguez et al., 2008; Gadadhar et al., 2011). In normal brain, neurogenesis is required for hippocampal synaptic plasticity and several forms of learning and memory (Shors et al., 2001; Leuner et al., 2004).

Inflammatory responses such as reactive astrocytes and activated microglia are widely seen in Alzheimer's brain (McGeer and McGeer, 1996). Astrocytes and microglia can produce inflammatory cytokines, reactive oxygen species, and other toxic materials (Rogers et al., 2002). In Alzheimer's brain, increased levels of pro-inflammatory cytokines and chemokines, such as interleukin(IL)-1β, IL-6, IL-8 and tumor necrosis factor-α, have been observed (Dickson et al., 1993; Griffin et al., 1995; Huell et al., 1995; Sokolova et al., 2009). Moreover, chronic neuroinflammation correlates with cognitive decline and brain atrophy in AD (Sokolova et al., 2009). Although the exact role of inflammation in neurodegeneration remains largely unknown, it is believed that uncontrolled neuroinflammation drives the chronic progression of neurodegenerative diseases (Gao and Hong, 2008).

Two previously published studies showed that forebrain excitatory neuron-specific *Dicer* cKO lines die either at early developmental stage (Davis et al., 2008) or early adulthood (Hébert et al., 2010). It is unlikely to use them for the study of adult neurogenesis. Here, we generated a mutant mouse in which Dicer is specifically inactivated in forebrain mature excitatory neurons from 1.5 to 2 months of age. Unlike the Davis et al. (2008) and the Hébert et al. (2010) lines, *Dicer* cKO in this study did not manifest early postnatal death. Whereas young *Dicer* cKO mice exhibited no abnormal neurogenesis, older cKO mice displayed dramatic neuron loss and neurogenesis impairment.

2. Materials and methods

2.1. Mice

Floxed *Dicer* mice ($Dicer^{f/f}$) and *CaMKIIα-Cre* transgenic (Tg) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). To generate mature neuron-specific *Dicer* cKO mice, $Dicer^{f/f}$ mice were crossed with *CamKIIα-Cre* to obtain $Dicer^{f/+}$*;CamKIIα-Cre*. The latter were bred to $Dicer^{f/f}$ to produce $Dicer^{f/f}$ (control), $Dicer^{f/+}$ (control), $Dicer^{f/+}$*;CamKIIα-Cre* (control) and $Dicer^{f/f}$*;CamKIIα-Cre* (cKO) for experiments. In this study, $Dicer^{f/f}$*;CaMKIIα-Cre* mice were referred to as *Dicer* cKO. Mice were bred in an SPF room of the core animal facility of the MARC. The room temperature was 25 °C and the light-cycle is automatically controlled (12 h for light and 12 h for dark). Mice had free access to food and water. The genetic background of the mice used in this study is C57BL/6. The number of animals used was 6–10/group/age.

2.2. Nissl staining

Sagittal brain sections (10 μm) were deparaffinized, ethanol dehydrated, and were then rinsed 5 min in distilled water. Sections were treated with 0.5% cresyl-violet for 10 min and then rinsed with distilled water three times. Sections were incubated in a solution containing 1% glacial acetic acid and 16% ethanol. Sections were dehydrated using an ascending series of ethanol (70%, 90%, 95% and 100%), and were then placed in toluene. Slides were coverslipped using neutral resin.

2.3. Immunohistochemistry

Brains were perfused with PBS, fixed in 4% paraformaldehyde, processed for paraffin embedding, and serially sectioned (10 μm). Sagittal sections were deparaffinized, ethanol dehydrated, and immunostained with monoclonal antibodies raised against NeuN (1:500, Millipore), microtubule-associated protein 2 (MAP2) (1:200; Sigma–Aldrich), SVP38 (1:500; Sigma–Aldrich), GFAP (1:500; Sigma–Aldrich), BrdU (1:200; Abcam), Iba1 (1:500; Wako), CC1 (1:200; Millipore) and DCX (1:200; Santa Cruz). For fluorescence immunostaining, brain sections were incubated with either Alexa Fluor 488 goat anti-mouse/anti-rabbit or Alexa Fluor 594 goat anti-mouse/anti-rabbit secondary antibodies (Invitrogen), and then analyzed with an Olympus confocal laser-scanning microscope.

2.4. Immunoblotting

Mice cortices were dissected and homogenized in cold radio immunoprecipitation assay lysis buffer [consisting of the following (in mM): 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] containing protease and phosphatase inhibitors (Thermo). Lysates were cleared by centrifugation (14,000 rpm for 20 min). Normalized volumes of samples (40 μg total protein) were resolved in 10% SDS-PAGE (invitrogen), transferred to nitrocellulose membrane. After blocking with 5% (w/v) dry milk for 1 h, membranes were probed with primary antibodies overnight and detected using infrared dye-coupled secondary antibodies (goat anti-rabbit IRdye800, goat anti-rabbit IRdye680, goat anti-mouse IRdye800 and goat anti-mouse IRdye680). Membranes were scanned and data were quantified using Odyssey Infrared Imaging System (Li-Cor). Primary antibodies used were as follows: anti-Dicer (1:200; Santa Cruz), anti-NeuN (1:500; Millipore), anti-GFAP (1:500; Sigma–Aldrich), anti-SVP38 (1:1000; Sigma–Aldrich), anti-PSD95 (1:1000; CST), anti-GPADH (1:10,000; Sigma–Aldrich) and anti-actin (1:10,000, SAB).

2.5. BrdU injection

BrdU is a thymidine analog and labels newly synthesized DNA. For the proliferation study, mice received a single dose of BrdU injection (Sigma, St. Louis, MO) intraperitoneally at the concentration of 100 mg/kg, and were sacrificed 24 h later. Paraffin-embedded brain sections were then prepared. Incorporated BrdU was detected by immuno-staining using an antibody against BrdU. The total number of BrdU positive (BrdU+) cells was counted using a stereological method. For the 1-month survival study, mice received intraperitoneal injection of BrdU once everyday for 3 consecutive days at the concentration of 100 mg/kg. Mice were sacrificed for brain sectioning 30 days after the final dose of BrdU.

2.6. TUNEL staining

The brain sections were blocked using 5% of goat serum for 30 min followed by the treatment of Fluorescein (Roche) at 37 °C for an hour (Wines-Samuelson et al., 2010; Tabuchi et al., 2009). The slides were then washed using TBS (tris-buffered saline) for three times. TUNEL staining was analyzed using an Olympus confocal laser scanning microscope. The total number of TUNEL+ cells in the cortex and the hippocampus were counted using a method reported previously (Tabuchi et al., 2009).

2.7. Stereological counting

The total numbers of BrdU+ cells in the SGZ and the SVZ were counted using a stereological method. Cells were counted from every twenty sections using a 40× objective throughout the entire SGZ and SVZ areas. Cells were counted within the granular cell layer (GCL) and adjacent SGZ up to a two-cell body-wide zone along the