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Molecules and Cells

Spontaneous Reactive Astrogliosis in the Dentate Gyrus of Bax-Deficient Mice

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Astrocytes play critical roles in many aspects of brain functions via modulation of neurotransmission, metabolism, and structural remodeling in response to physiological or pathological stimuli. Activation of astrocytes is a common phenomenon in many brain pathologies such as stroke, trauma, and neurodegenerative diseases. In this study, we found that gene deletion of the pro-apoptotic gene Bax (Bax-knockout) resulted in a spontaneous reactive astrogliosis in the dentate gyrus, as evidenced by the increased number/volume of astrocytes and cytoplasmic localization of the Olig2 protein. On the other hand, there was no evidence for microglial activation in the dentate gyrus of Bax-knockout mice. Previously, we reported that Bax-knockout mice failed to execute programmed cell death of adult-produced neurons, but the surplus neurons eventually impaired normal synaptic connections and dendritic arborization of dentate gyrus neurons. Therefore, we propose that the reactive astrocytes in the Bax-knockout mice may play a role in tissue remodeling of the dentate gyrus following a failure in the programmed cell death of adult-produced neurons.

INTRODUCTION

The adult brain maintains neural stem/progenitor cells that can differentiate into all major neural cell types such as neurons, astrocytes, and oligodendrocytes (Gage, 1995; Rietze et al., 2001). Neural stem/progenitor cells are located in specific neurogenic niches in the subgranular zone of the hippocampal dentate gyrus (DG) and anterior subventricular zone of the lateral ventricle. In addition to these multipotent neural stem cells, glial progenitor cells, which generate oligodendrocytes and astrocytes, are more widely distributed in the adult rodent brain (Nishiyama et al., 1999). Both neural stem cells and glial progenitor cells express the Olig2 transcription factor, and Olig2 expression or its subcellular distribution was markedly modified during cell type specification/differentiation (Menn et al., 2006; Vassilev et al., 1992). For instance, the specification of neuronal lineage cells requires the suppression of Olig2 expression, whereas glial lineage cells maintain Olig2 expression (Fukuda et al., 2004; Marshall et al., 2005). Although Olig2-expressing progenitors normally produce oligodendrocytes, they can also produce reactive astrocytes by receiving injury-induced signals (Magnus et al., 2007; Ono et al., 2009; Zhao et al., 2009). During reactive astrogliosis, Olig2 proteins in the nuclei are rapidly translocated into the cytoplasm, presumably for rapid suppression of nuclear Olig2 function. Reactive astrocytes are involved in many aspects of injury responses, including glial scar formation, promotion/suppression of regeneration, and remodeling of synapses (Sofroniew, 2009; Zhang et al., 2010).

Recently, we found that mice with gene deletion of the pro-apoptotic gene Bax (Bax-KO) exhibited an impaired hippocampus-dependent synaptic plasticity and associated learning behavior (Kim et al., 2009; Sun et al., 2004). After initial production, approximately 50% of the newly produced DG neurons undergo Bax-dependent programmed cell death (PCD) (Sun et al., 2004). Because Bax-KO mice cannot eliminate these surplus DG neurons, Bax-KO mice exhibited an age-dependent increase in the number of DG neurons. Importantly, in spite of the continuous addition of new DG neuron, the numbers of other synaptically connecting efferent and afferent neurons remained constant, which in turn induced the modification/impairment of efferent (from entorhinal cortex to DG) and afferent (from DG to CA3 hippocampus) synaptic connections. Considering the close communication of glial cells with neighboring neurons, these changes in neuronal populations may give rise to the secondary responses of glial cells. In this study, we found that a subset of astrocytes in the molecular layer of the Bax-KO DG exhibited hallmarks for reactive astrogliosis such as cytoplasmic localization of Olig2. Although it has been well documented that neuronal degeneration triggers glial responses, our current observation suggests that the failure in the removal of newly produced neurons also promotes the adaptive glial responses such as reactive astrogliosis.

MATERIALS AND METHODS

Animals

Bax-KO mice were maintained as heterozygotes on a C57Bl/6 background. Homozygous Bax-KO mice were obtained by breeding heterozygous mice, and the genotypes of the sibling animals were individually assessed by polymerase chain reaction (PCR) as previously described (Kim et al., 2009).

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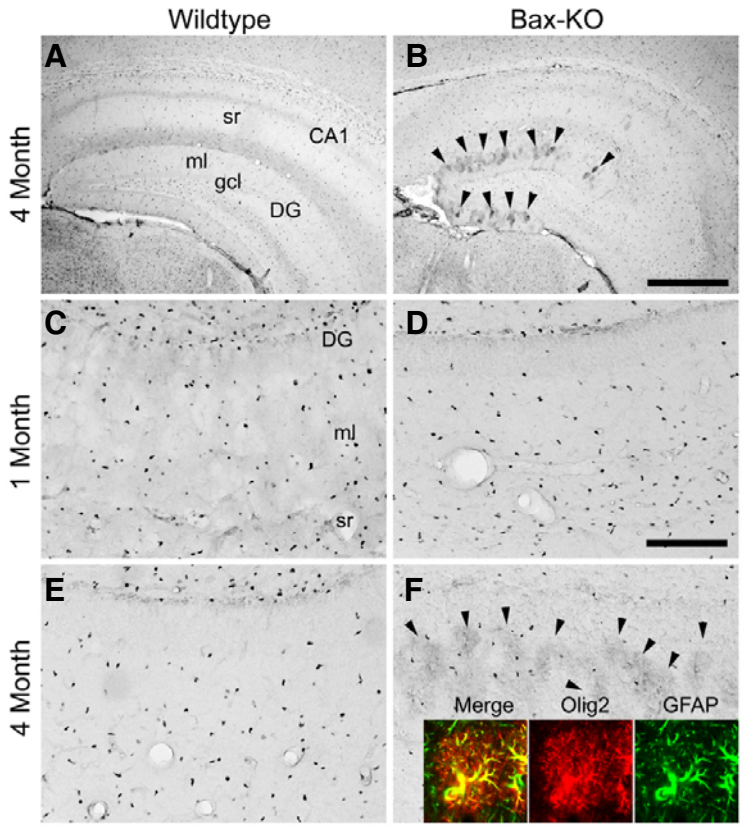

Fig. 1. Localization of Olig2-IR in 1- (C, D) and 4-month-old (A, B, E, and F) WT (A, C, and E) and Bax-KO (B, D, and F) hippocampus. In the WT and 1-month-old Bax-KO hippocampus, Olig2-IR was exclusively found in the nuclei. In contrast, cytoplasmic Olig2-IR was found in the molecular layer (ml) of 4-month-old Bax-KO DG (arrows in B and F). Insets in F show double immunofluorescence labeling (left) of Olig2 (middle, red) and GFAP (right, green). Bar = 100 μm for A and B, and 25 μm for (C-F). Abbreviations are DG, dentate gyrus; sr, stratum radiatum; ml, molecular layer of dentate gyrus; gcl, granule cell layer; CA1, CA1 field of the hippocampus.

Immunohistochemistry

Mice were cardiac perfused with fixative (4% paraformaldehyde), and their brains were isolated. Following post-fixation overnight, brains were cryoprotected in 30% sucrose solution overnight, sectioned serially (40 μm, coronal cut) on a cryostat, and stored in 50% glycerol/50% phosphate-buffered saline (PBS) solution at -20°C until use. For immunofluorescence labeling, sections were blocked with 10% bovine serum albumin and 0.1% Triton X-100 in PBS for 30 min. Primary antibodies were applied overnight. After washes with PBS, appropriate secondary antibodies were applied for 30 min. Subsequently, sections were washed, mounted, and observed by confocal microscopy (Zeiss LSM510, Germany). For 3,3′-diaminobenzidine (DAB) labeling, sections were rinsed with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity (Lee et al., 2008). After blocking with 3% bovine serum albumin and 0.1% Triton X-100 in PBS for 30 min, primary antibodies were applied overnight. After washes with PBS, biotinylated secondary antibodies (Amersham Biosciences, USA) were applied for 30 min, and the signals were visualized using Vectastain Elite ABC kit and DAB solution (Vector Laboratories, USA). Antibodies used in this study were Olig2 (OA-520; 1:200; IBL, Japan), GFAP (MAB360; 1:1,000; Millipore, USA), Iba1 (#019-19741; 1:1000; Wako, Japan), PSA-NCAM (#MAB5324; 1:500; Millipore), and DCX (Sc-8066; 1:1000; Santa Cruz Biotechnology, USA).

Quantification

To quantify the number of labeled cells, images from sections containing dorsal DG were captured using a computerized image program. At least two different sections per animal were analyzed, and the mean cell number in the molecular layer of the DG inferior blade was obtained. Statistical significance of differences between the groups was evaluated by Student's *t*-test comparisons. All the analyses were carried out with SPSS software, and all values are presented as mean ± standard error of the mean (SEM).

RESULTS

Cytoplasmic localization of Olig2 in a subset of astrocytes in the Bax-KO DG

Immunostaining of Olig2 in 4-month-old wild-type (WT) mouse brains demonstrated that cells with nuclear Olig2-immunoreactivity (IR) were widely distributed in most brain regions, including the hippocampal formation (Fig. 1). Cells with nuclear Olig2-IR were similarly distributed in the hippocampus of Bax-KO littermates, but a subset of cells in the molecular layer of the DG also exhibited cytoplasmic Olig2-IR (Figs. 1B and 1F, inset). Quantification of Olig2-IR cells showed that the total numbers of nuclear Olig2+ cells were similar in the DG and CA1 region of WT and Bax-KO mice (WT DG, $122.8 \times 10^3 \pm 23.9 \times 10^3$ cells/mm^2, n = 4; Bax-KO DG, $115.0 \times 10^3 \pm 28.1 \times 10^3$ cells/mm^2, n = 4; WT CA1, $95.1 \times 10^3 \pm 20.7 \times 10^3$ cells/mm^2, n = 4; Bax-KO CA1, $124.3 \times 10^3 \pm 14.0 \times 10^3$ cells/mm^2, n = 4). Cells with cytoplasmic Olig2-IR were exclusively localized in the Bax-KO DG and comprised 27.0% of the total Olig2+ cells found ($31.0 \times 10^3 \pm 8.1 \times 10^3$ cells/mm^2, n = 4). Interestingly, cytoplasmic Olig2-IR was not observed in 1-month-old Bax-KO mice (Fig. 1D), and only a small number was found in the 2-month-old Bax-KO DG (data not shown), suggesting that the cytoplasmic localization of Olig2 occurs after 1 month of age, and their numbers increase age-dependently. It has been reported that reactive astrocytes can be produced from Olig2+ progenitor cells, and nuclear

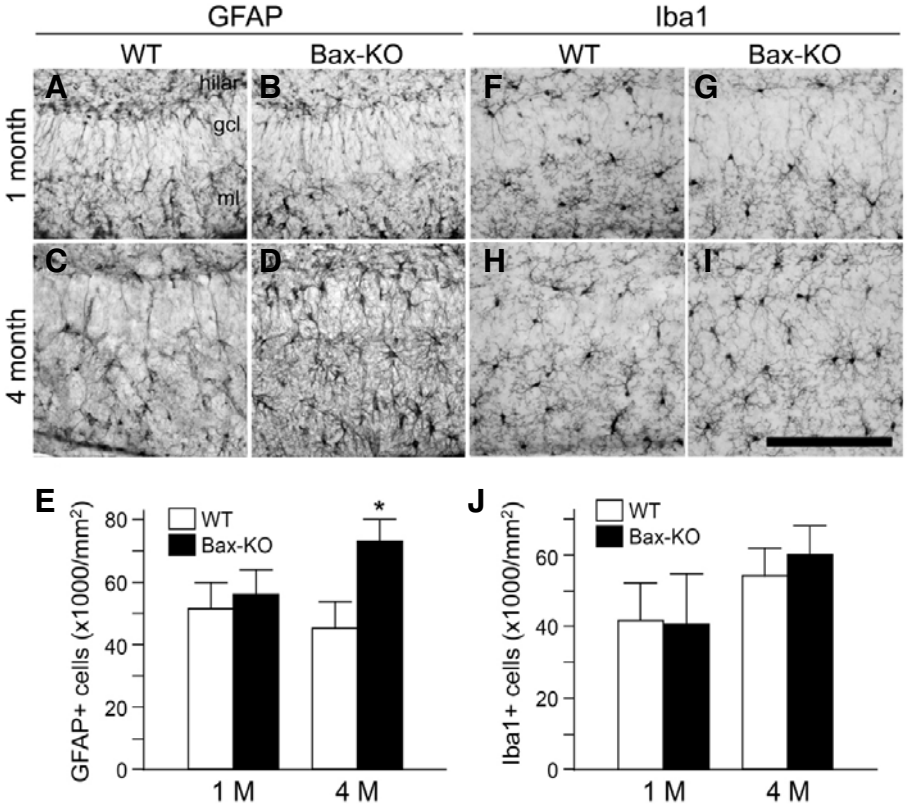

Fig. 2. Distribution of GFAP-labeled astrocytes (A-D) and Iba1-labeled microglia (F-I) in 1- (A, B, F, and G) and 4-month-old (C, D, H, and I) WT (A, C, F, and H) and Bax-KO (B, D, G, and I) DG. Bar = 20 μm for (A-D) and (F-I). Quantification of GFAP+ cells (E) and Iba1+ cells (J) are shown. N = 3, DATA = Mean ± SEM. *P < 0.05 in Student's t-test comparison.

Olig2 rapidly translocates to the cytoplasm during the differentiation process (Magnus et al., 2007; Zhao et al., 2009). Furthermore, highly branched morphology with distinct round boundaries suggested that they were astrocytes. By double-fluorescence labeling of Olig2 with GFAP, we confirmed that the cells with cytoplasmic Olig2-IR were GFAP-expressing astrocytes (Fig. 1F, inset).

Changes in astrocytes and microglia in the Bax-KO DG

Cytoplasmic localization of Olig2-IR is known to be associated with reactive astrogliosis (Zhao et al., 2009). Next, we further addressed the distribution and morphology of GFAP-expressing astrocytes in WT and Bax-KO mice (Figs. 2A-2E). Compared to WT, 4-month-old Bax-KO mice exhibited abundant, thick process-containing astrocytes in the DG, which is a hallmark of reactive astrogliosis. Quantification of astrocytes in the DG demonstrated a significant increase in the number of astrocytes in the 4-month-old Bax-KO DG (Fig. 2E). In contrast, the number and distribution of astrocytes were normal in 1-month-old Bax-KO mice, suggesting that the mild reactive astrogliosis is an age-dependent process (Figs. 2A-2E). Reactive astrogliosis is often associated with brain damage and subsequent microglial activation. However, we failed to observe activated amoeboid microglia in both the WT and Bax-KO DG, and the numbers of microglia in the DG were similar in 1- or 4-month-old WT and Bax-KO mice (Figs. 2F-2J), suggesting that the reactive astrogliosis in the Bax-KO DG was not associated with microglial activation.

Astrocytic disorganization and ectopic neuroblast migration in the Bax-KO DG

Because reactive astrogliosis may be related to the increased survival of newly generated granule cells in the 4-month-old Bax-KO DG, we examined the relationship between immature neuroblasts and astrocytes by double immunofluorescence labeling (Figs. 3A-3E). In WT mice, most PSA-NCAM-labeled immature neuroblasts were surrounded by astroglial processes, and some radial processes of migrating neuroblasts were associated with radial glial fibers (Figs. 3A-3C, insets). Similarly, increased numbers of neuroblasts in the Bax-KO DG were also tightly associated with an increased number of astrocytes. On the other hand, neuroblasts were not associated with microglia in both the WT and Bax-KO DG (Figs. 3G-3L).

DISCUSSION

In this study, we present evidence for spontaneous astrogliosis in the Bax-KO DG, including an increased number of astrocytes, astrocytic hypertrophy, and cytoplasmic localization of Olig2 protein. It has been reported that Olig2-expressing oligodendrocyte progenitor cells can produce reactive astrocytes in response to brain injury, and cytosolic translocation of Olig2 protein has been observed during astrocytic differentiation (Magnus et al., 2007; Zhao et al., 2009). In addition, cytoplasmic Olig2-IR and reactive astrogliosis suggest that the increased number of astrocytes in the Bax-KO DG originate from Olig2-expressing glial progenitors, but not from neural progenitors localized in the subgranular zone. Consistent with our previous result, we demonstrated that the fate of neurogenesis and gliogenesis from the subgranular zone is not affected in Bax-KO mice (Sun et al., 2004).

Previously, we reported the disruption of astrocyte development in the rostral migratory stream (RMS) in Bax-KO mice (Kim et al., 2007), which may be due to the elimination of astrocytes by the excess migrating neuroblasts (Kaneko et al., 2010). However, we failed to identify cells with cytoplasmic Olig2-IR in

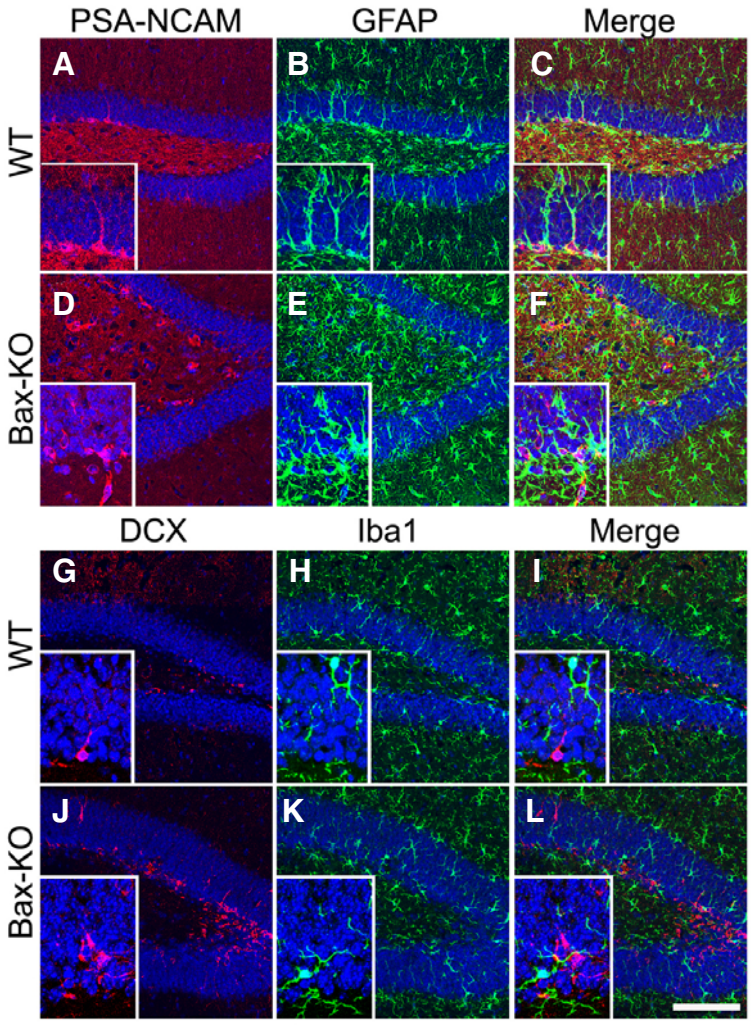

Fig. 3. (A-F) Distribution of PSA-NCAM-labeled neuroblasts (A, D) and GFAP-labeled astrocytes (B, E) in WT (A-C) and Bax-KO (D-F) DG. (C) and (F) show merged images. Insets demonstrate large magnification image of cell exhibiting close association of neuroblasts and astrocytes. (G-L) Distribution of DCX-labeled neuroblasts (G, J) and Iba1-labeled microglia (H, K) in WT (A-C) and Bax-KO (D-F) DG. (C) and (F) show merged images. Insets demonstrate large magnification image of subgranular zone. Bar = 10 μm for A-F.

the RMS (data not shown), suggesting that the mechanisms of astrocytic changes in the RMS and DG are different. Because Bax is critical for the execution of cell elimination after initial genesis of new neurons in the adult brain, Bax-KO mice exhibited an age-dependent accumulation of extra neurons in the DG (Sun et al., 2004). Interestingly, reactive astrogliosis was selectively observed in the Bax-KO DG at 4 months of age, when accumulation of adult-produced neurons became severe. Therefore, astrogliosis may be closely related to the accumulation of adult-produced neurons in the Bax-KO DG. Considering that we used germ-line Bax-KO mice, which lack the Bax gene in all astrocytes at any age, selective astrogliosis in the 4-month-old Bax-KO DG suggests that an environmental cue is crucial for the astrogliosis and thus excludes the possibility that Bax is directly involved in the reactive astroglial process in a cell-autonomous manner.

Reactive astrocytes are normally generated in response to brain injury, with a considerable loss of neurons (Sofroniew, 2009; Zhang et al., 2010). However, the Bax-KO DG contained little or no dying neurons. Moreover, the Bax-KO DG did not exhibit any sign of microglial activation, and all microglia maintained their quiescent and ramified morphology. Therefore, this result suggests that the reactive astrogliosis in the Bax-KO DG is not associated with neuronal loss and subsequent microglial activation. Although cell death is completely prevented in the Bax-KO DG, we have previously found that Bax-KO DG neurons have fewer dendritic branches (Kim et al., 2009), suggesting that there may be an increased level of dendritic/synapse pruning in the Bax-KO DG. Pruning is an elimination process to remove extra neuronal branches or synapses and can be mediated by astrocytes and/or microglia (Allen and Barres, 2005; Stevens, 2008). However, microglia were not closely associated with immature DG neurons, and only astrocytes were activated in the Bax-KO DG, suggesting that reactive astrocytes are responsible for the dendritic remodeling in Bax-KO mice. Reactive astrocytes near the immature neuroblasts may also influence other aspects of neuronal development, such as maintaining progenitor cell niches (Alvarez-Buylla and Lim, 2004) and neuronal migration (Nacher et al., 2001). In particular, we previously found that some neuroblasts ectopically migrate toward the hilar in Bax-KO mice (Sun et al., 2004), and such migration defects may be associated with reactive astrogliosis and disrupted radial glia arrangement in the Bax-KO granule cell layer.

In conclusion, we postulate that the successive tissue remodeling (increased survival of new neurons → activation of astrocytes → increased dendrite pruning/impairment of neuronal migration) may be an adaptive response following a failure of PCD in the adult mouse brain.

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