Brain & Development 36 (2014) 661–669

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Disruption of *Aspm* causes microcephaly with abnormal neuronal differentiation

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Received 14 June 2013; received in revised form 11 October 2013; accepted 13 October 2013

Abstract

Aims: A number of *ASPM* mutations have been detected in primary microcephaly patients. In order to evaluate the function of ASPM in brain development, we generated model animals of human autosomal recessive primary microcephaly-5 (MCPH5). *Methods:* In the *Aspm* knock-out mice, the exon 2–3 of the *Aspm* gene was encompassed by a pair of loxP signals so that cre-recombinase activity switched the allele from wild-type to null zygotes as frequently, as expected from the Mendelian inheritance. We precisely analyzed the brains of adults and fetuses using immunohistochemistry and morphometry. *Results:* The adult brains of the $Aspm^{-/-}$ mice were smaller, especially in the cerebrum. In the barrel field of the somatosensory cortex, layer I was significantly thicker, whereas layer VI was significantly thinner in $Aspm^{-/-}$ mice, compared with $Aspm^{+/+}$ mice. The total number of cells and the thickness of the cortical plate at embryonic day 16.5 was significantly decreased in $Aspm^{-/-}$ mice, compared with $Aspm^{+/+}$ mice. Furthermore, the expression of transcription factors, such as Tbr1 and Satb2, was significantly increased in the subplate of the $Aspm^{-/-}$ mice. *Conclusions:* The results suggested that *Aspm* is essential to the proliferation and differentiation of neural stem/progenitor cells. The *Aspm* gene loss model provided a novel pathogenetic insight into acquired microcephaly, which can be caused by *in utero* exposure to both known and unknown teratogens.

Keywords: *ASPM*; Knock-out mice; Microcephaly; Cortex; Differentiation

1. Introduction

The human *ASPM* (*abnormal spindle-like microcephaly-associated*) gene was identified at the locus of MCPH5, the most common type of autosomal recessive primary microcephaly, and it was first cloned by Bond et al. [1]. This gene consists of 28 exons encoding a large peptide of 3477 amino acids, with four distinct domains; putative microtubule-binding, calponin-homology, multiple IQ calmodulin-binding, and terminal regions. The murine ortholog gene was identified as *calmodulin binding protein* 1 (*calmbp1*). Murine *Aspm* also consists of 28 exons and the deduced protein structure was similar to human *ASPM* except for the size of the repetitive IQ motifs.

MCPH5 is characterized by microcephaly present at birth and non-progressive mental retardation [1–6]. The microcephaly is the result of a small, but architecturally normal brain, and it is the cerebral cortex that shows the greatest reduction in size [7]. It has been proposed that the *Aspm* gene is the major determinant of

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http://dx.doi.org/10.1016/j.braindev.2013.10.006

cerebral cortical size among primates, including humans [8–10]. The function of the Aspm protein in neural progenitor cell expansion, as well as its localization in the mitotic spindle and centrosome, suggest that it regulates brain development by a cell division-related mechanism [11–16]. In addition, a loss of Aspm proteins also causes a massive loss of germ cells, resulting in a severe reduction in the size of the testes and ovaries, accompanied by reduced fertility [17].

The results of anthropological investigations suggest that the ASPM protein plays some roles in the determination of the size of the human cerebral cortex, from an evolutionary viewpoint [8–10]. Drosophila *asp* mutants have shown premature condensation of mitosis in affected brains [18]. Knocking-down of murine *Aspm in utero* demonstrated that the transient reduction of Aspm causes loss of symmetric division of neuroepithelial cells, which is critical for the following neural cell type differentiation [13]. Thus, brain size seems highly associated with the mitotic activity of Aspm selectively in the neural stem cells and progenitors, although the molecular basis for this selectivity remains to be clarified.

In this study, we showed aberrant brain development in a murine model for human *ASPM* deficiency. The adult mice with knocked-down *Aspm* demonstrated decreased brain weight and volume, especially in the neocortex, where cortical layer VI was thinner in the somatosensoty barrel field, compared with $Aspm^{+/+}$ brains. Furthermore, the expression pattern of the layer-specific transcription factors was disrupted in fetal brains at embryonic day 16.5, suggesting that the loss of Aspm caused not only proliferation disorder in earlier embryonic stage, but also disrupted cell fate and differentiation of the cortical neurons.

2. Materials and methods

2.1. Tissue preparation and histopathological examination

Adult mice were perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS. The brains and testes were removed, post-fixed, and paraffin-embedded coronal sections were stained with hematoxylin–eosin ($Aspm^{-/-}$: $n = 6$, $Aspm^{+/-}$: $n = 6$, $Aspm^{+/+}$: $n = 5$). The fetal brains at E16.5 were dissected and fixed overnight with 4% paraformaldehyde, followed by cryoprotection in 20% sucrose at 4 °C ($Aspm^{-/-}$: $n = 6$, $Aspm^{+/-}$: $n = 6$, $Aspm^{+/+}$: $n = 6$). Fixed brains were embedded in 4% CMC compound and frozen in powdered dry ice. Serial coronal sections were prepared using a cryomicrotome (LEICA CM1850, Leica, Japan) at 20 μm-thickness. After blocking with 10% goat serum, 1% BSA, and 0.01% Triton X-100 (Sigma–Aldrich, Japan) in 0.1 M PBS, the sections were incubated in primary antibody at 4 °C overnight; Aspm (rabbit polyclonal, produced by Fujimori A: 1:100), Satb2 (mouse monoclonal, ab51502, abcam, MA, USA: 1:50), Ctip2 (rat monoclonal, ab18465, abcam, MA, USA: 1:500), Tbr1 (rabbit monoclonal, ab31940, abcam, MA, USA: 1:500), diluted in 0.1% Triton X-100 and PBS. The sections were rinsed with PBS and then incubated in the secondary antibody (Alexa 647-conjugated goat anti-mouse IgG, Alexa 647-conjugated goat anti-rabbit IgG, Alexa 647-conjugated goat anti-rat IgG, 1:300, Invitrogen, Japan) and counterstained by YOYO-1 iodide (Invitrogen, Japan). The sections were then observed under a confocal microscope (FluoView FV1000 Confocal Microscope; OLYMPUS, Japan).

2.2. Image analysis

2.2.1. Cortical layer ratio of adult brain

Three serial coronal sections from the bregma −0.10 mm to the bregma −1.58 mm were selected [19]. The motor cortex (M1), sensory cortex (S1) of forelimb region and the barrel field of the somatosensory cortex-1 (S1BF) were analyzed at the bregma −0.10 mm and the sensory cortex (S1) of trunk region and barrel field of the somatosensory cortex-2 (S1BF) were analyzed at the bregma −1.58 mm. The thickness of cortex and each cortical layer, such as layer I, II + III, IV, V, and VI, was measured in brain regions M1, S1, and S1BF using Image-Pro Analyzer 7.0 J (Fig. 3B). Briefly, the vertical column was drawn from the cortical surface to the border of the cortex and white matter and the thickness of each layer was calculated according to the histological features. In order to avoid artificial factors induced by tissue processing, the ratios of each cortical layer were obtained, normalized by the total cortical thickness. Five columns were set in each analyzed area per section and three serial sections were analyzed (15 data sets for each brain area). Finally, we statistically analyzed the ratios in M1, and S1 (forelimb + trunk region) and S1BF(1 + 2) from $Aspm^{-/-}$ ($n = 6$), $Aspm^{+/-}$ ($n = 6$), and $Aspm^{+/+}$($n = 5$) mice.

2.2.2. Transcription factor-expressing cells in the fetal cortical plate and subplate

In order to evaluate the areas that were immunoreactive for each transcription factor in the fetal brains, we measured every pixel showing immunofluorescence after binarization of digital images by the threshold method (Otsu). We counted the total nuclear area (N, μm^2), immunoreactive for YOYO, and the transcription factor-expressing area (TR, μm^2) of the cortical plate and the transcription factor-expressing area in the subplate at the level of GD16, coronal 11, and coronal 12 from the atlas of the prenatal mouse brain [20]. The counting frame of 404379 μm^2 (635.9 × 635.9 μm) was placed randomly three times on the image analyzer system