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Camk2a-Cre-Mediated Conditional Deletion of Chromatin Remodeler Brg1 Causes Perinatal Hydrocephalus

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Abstract

Mammalian SWI/SNF-like BAF chromatin remodeling complexes are essential for many aspects of neural development. Mutations in the genes encoding the core subunit Brg1/SmarcA4 or other complex components cause neurodevelopmental diseases and are associated with autism. Congenital hydrocephalus is a serious brain disorder often experienced by these patients. We report a role of Brg1 in the pathogenesis of hydrocephalus disorder. We discovered an unexpected early activity of mouse Camk2a-Cre transgene, which mediates *Brg1* deletion in a subset of forebrain neurons beginning in the late embryonic stage. *Brg1* deletion in these neurons led to severe congenital hydrocephalus with enlargement of the lateral ventricles and attenuation of the cerebral cortex. The *Brg1*-deficient mice had significantly smaller subcommissural organs and narrower Sylvian aqueducts than mice that express normal levels of Brg1. Effects were non-cell autonomous and may be responsible for the development of the congenital hydrocephalus phenotype. Our study provides evidence indicating that abnormalities in Brg1 function result in defects associated with neurodevelopmental disorders and autism.

Keywords

Brg1/SmarcA4; Camk2a-Cre; hydrocephalus; autism; aqueduct; SCO

Introduction

Hydrocephalus is a severe neurological disorder characterized by an excess of cerebrospinal fluid (CSF) in the brain and dilatation of the cerebral ventricles. The causes of hydrocephalus are not well understood, although accumulating evidence suggests that genetic factors are critical to the pathogenesis of hydrocephalus [27]. Congenital hydrocephalus is the most common type and aqueductal stenosis is the major contributing

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factor in congenital hydrocephalus with a prevalence of 0.1-0.3% of all live births[18]. Intellectual disability disorders and autism are associated with hydrocephalus. For example, approximately one quarter of the children with hydrocephalus in a population-based study had been diagnosed with an autism spectrum disorder (ASD)[5], and 2% of the children with ASD [2]have hydrocephalus, compared with fewer than one per 1,000 in the general population [19]. A number of genes, including several transcription factors, cause congenital hydrocephalus in animal studies[12, 20, 25, 33]; however, the molecular mechanisms that underlie the phenotype have remained elusive and it is not clear that the identified genes are associated with human hydrocephalus.

The mammalian SWI/SNF-like BAF complexes are ATP-dependent chromatin remodeling complexes that use energy generated from ATP hydrolysis to alter DNA-nucleosome contacts[1, 30]. Chromatin remodeling complexes are important for development and in cancer and stem cell biology[8]. Recent human exome sequencing and genome-wide association studies implicated mutations in genes encoding the core ATPase subunit Brg1 and its associated proteins cause Coffin-Siris syndrome and Nicolaides-Baraitser syndrome [22, 26, 28]. Patients with these diseases have autistic symptoms such as intellectual disability and delayed speech [6, 10]. Some patients also develop the Dandy-Walker brain abnormality, which is hydrocephalus in the cerebellum region[11]. In addition, *de novo* functional mutations of genes encoding several BAF subunits are observed in autism patients with relatively high frequencies [4, 7, 9, 17]. These data suggest a connection between BAF complexes and autism-related hydrocephalus.

Developmental stage-specific genetic deletion of BAF subunits in mice revealed that the Brg1 and BAF complex is critical at multiple steps during neural development. Nestin-Cre-mediated *Brg1* deletion showed that Brg1 is essential in neural stem/progenitor cell self-renewal, proliferation, and differentiation[13]. These mice die at birth and have thin cortices but not hydrocephalus. Deletion of the gene encoding a neuron-specific BAF subunit, BAF53b, demonstrated a critical function of BAF53b in dendritic development and learning and memory[29, 31]. Deletion of *Brg1* and genes encoding other BAF subunits in cerebellum neural precursors impaired progenitor proliferation and cerebellum growth[16, 32]. These genetic studies provide insights to BAF functions in neurological diseases. However, it is not clear whether BAF plays a role in hydrocephalus pathogenesis.

In this study, we used the widely used postnatal forebrain neuron-specific *Camk2a-Cre* system to delete *Brg1*. Unexpectedly, we observed an embryonic activity of the *Camk2a-Cre* transgene. The *Camk2a-Cre*-mediated deletion of *Brg1* in forebrain neurons led to abnormal formation of the subcommissural organ (SOC) and narrowing of the aqueduct in a non-cell-autonomous manner, a possible cause of the congenital hydrocephalus phenotype. This study reveals a surprisingly early activity of *Camk2a-Cre* and suggests that loss of functional Brg1 lead to hydrocephalus and neurodevelopmental diseases.

Materials and Methods

Mice

Brg1^{F/F} mice [24] and *Camk2a-Cre* [15] mice were kindly provided by Drs. Pierre Chambon (IGBMC, France) and Rudiger Klein (Max-Planck Institute, Germany) respectively. The *Ai9* mice [14] were purchased from The Jackson Laboratory. These mice are maintained on a mixed genetic background at UT Southwestern Medical Center Animal Facility. All the procedures performed were in accordance with the IACUC approved protocols and rules.

Genotyping of mice

The mouse genotypes were analyzed with polymerase chain reaction. Protease-digested mouse tail samples were amplified 30 cycles (30 seconds at 94 °C, 45 second at 55 °C, and 45 seconds at 72 °C) on a thermal cycler with indicated primers. The *Camk2a*-specific primers are 5'-GTTCTCCGTTTGCCTCAG-3' and 5'-CTCATCACTCGTTGCATCG-3', which are designed to match sequences in the *Camk2a* enhancer and in the *Cre* coding region, respectively. The *Cre*-specific primers are 5'-TCGATGCAACGAGTGATGAG-3' and 5'-TCCATGAGTGAACGAACCTG-3'.

Histology and immunofluorescence staining

Animals were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in PBS. Brains were postfixed in 4% PFA overnight at 4°C, washed in PBS, dehydrated, and paraffin embedded using standard protocols. Samples were sectioned at 7 µm on a sliding microtome and mounted on Colorfrost Plus slides. Haematoxylin and eosin (H&E) staining and immunofluorescence staining were performed on paraffin sections. Antibodies used were against Brg1 (G-7, Santa Cruz) and RFP (Rockland). The images were visualized using an Olympus BX50 microscope.

Cell quantification

Cells in a size-defined area around the aqueduct were quantified using ImageJ software. Comparable sections (5 sections each, n=4 mice) at the same anteroposterior levels were stained with DAPI and photographed using 10x magnification lens. Average cell densities were shown. Student's t-test was used to assess statistical significance between groups. A *p* value of <0.05 was considered significant.

Results

Camk2a-Cre-mediated *Brg1* deletion causes perinatal hydrocephalus

To determine the function of Brg1 in postnatal neuron development, we crossed mice carrying the *Brg1^{F/F}* conditional allele with the widely used *Camk2a-Cre* (line 93) mouse [15]. This *Camk2a-Cre* transgenic line was generated by inserting the *Cre* coding region downstream of the 8.5kb *Camk2a* gene enhancer element. It is generally thought that the *Camk2a-Cre* activity peaks in forebrain pyramidal neurons in postnatal mouse brain and is not active in the embryos [3]. Unexpectedly, we observed an early hydrocephalus phenotype from *Camk2a-Cre Brg1^{F/F}* mice.

Camk2a-Cre Brg1^{F/F} mice were born with the expected Mendelian ratio but died within 2 months of age. Beginning soon after birth, homozygous *Brg1*-mutant pups showed dome-shaped heads, a sign of hydrocephalus (Figure 1A). The brains from *Brg1*-deficient mice accumulated cerebrospinal fluid in the lateral ventricles, which collapsed during brain isolation. Analysis of gross brain morphologies indicated enlarged hemispheres and compressed olfactory bulbs relative to those of *Brg1^{F/F}* mice (Fig. 1B). The H&E staining of P11 brain sections showed that the lateral ventricles were massively dilated and the cerebral cortex was remarkably thin (Figure 1C). Moreover, in *Camk2a-Cre Brg1^{F/F}* mice, we observed a significant reduction of the corpus callosum and decreased size of the hippocampus compared to control mice. The mid-hind brain regions are grossly similar between control and *Brg1* mutant mice. To our surprise, the hydrocephalus phenotype was apparent even at birth. P0 brains had enlarged lateral ventricles and thinning of the cortex (Figure 1C). This result indicates that the hydrocephalus phenotype of *Camk2a-Cre Brg1^{F/F}* mice was caused by defects starting from embryonic stage.

***Camk2a-Cre* activities in the forebrain during perinatal development**

Camk2a-Cre was previously reported to act mainly in postnatal forebrain neurons with peak expression 2-3 weeks after birth [3]. The phenotypes of *Camk2a-Cre Brg1^{F/F}* mice were observed at P0; therefore, we sought to verify the *Camk2a-Cre* activity. Genotyping using the primers spanning the *Camk2a* enhancer and the *Cre* coding region confirmed the identity of the *Camk2a-Cre* transgene in the *Camk2a-Cre Brg1^{F/F}* mice; an *Emx1-Cre* transgene produced no signals with this set of primers (Figure 2A). To determine the activity of *Camk2a-Cre*, we crossed the *Camk2a-Cre* mice with the Ai9 Cre reporter mouse [14]. The Ai9 mouse has a tdTomato expression cassette inserted in the *Rosa26* locus. A stop signal flanked by two LoxP sites was placed upstream of the tdTomato coding region to repress tdTomato expression [14]. Cre activity results in deletion of the stop signal and enables tdTomato expression. In *Camk2a-Cre Ai9* brain, with an antibody against RFP and its variant, we were able to detect tdTomato signals as early as E17.5 (Figure 2B). As previously described [3], the Cre activity was evident in the forebrain, including cerebrum, striatum, and hypothalamus, but was mostly absent from mid-hind brain regions. At E17.5 and P0, tdTomato-positive cells were observed in the differentiated regions as well as in the ventricular zone where neural progenitors reside (Figure 2B). These results indicate that the Ai9 line is a sensitive Cre reporter, and our data indicate a previously unreported early activity of the Cre system in the *Camk2a-Cre* (line 93) mice.

Camk2a-Cre Brg1^{F/F} mice displayed progressive *Brg1* deletion from forebrain neurons such as those in the cortex and hippocampus (Figure 2C), where endogenous *Camk2a* genes are strongly expressed as reported previously [23]. As early as E17.5 and at P0, *Brg1* deletion was detected in regions such as dentate gyrus and cortex. At P11 *Brg1* deficiency in a subset of neurons in the cortex, CA1, and dentate gyrus regions in the hippocampus became apparent. These data suggest that an early *Camk2a-Cre* activity mediated *Brg1* deletion in a subset of forebrain neurons caused perinatal hydrocephalus phenotype.

The non-cell autonomous effects of *Brg1* deletion on the stenosis of the aqueduct

The primary cause of congenital hydrocephalus is blockage of cerebral spinal fluid flow due to aqueduct narrowing. To determine whether the hydrocephalus observed in *Camk2a-Cre Brg1^{F/F}* mice resulted from the abnormal aqueduct structure, brains of *Brg1* mutant and control littermates were sectioned to compare the aqueduct of Sylvius and surrounding tissues. At the entrance of the aqueduct, the subcommissural organ (SCO), which is important for CSF flow and associated with hydrocephalus pathogenesis [18], was significantly smaller in *Brg1* mutant than in the control brains (Figure 3A). The aqueduct in both rostral and caudal regions was also narrower in *Brg1* mutant than in the control mice. The abnormalities in SCO and aqueduct likely resulted in the hydrocephalus phenotype. Interestingly, *Camk2a-Cre* was not expressed in the midbrain region where the aqueduct resides (Figure 2B). We confirmed the presence of *Brg1* in the SCO cells, ependymal cells lining the aqueduct, and the neural cells in the surrounding tissues (Figure 3B). Therefore, *Brg1* deletion in a subset of forebrain neurons mediated by *Camk2a-Cre* played a non-cell autonomous role in regulating formation of the SCO and aqueduct structures. A close examination indicated that there was a significant increase in the densities of the ependymal cells lining the aqueduct in *Brg1*-mutant mice (Figure 3C, 3D). In the neighboring regions, there was also a 60% increase of cell densities in the *Brg1* mutant brains compared to controls (Figure 3C, 3D). The increased cellularity surrounding the aqueduct in *Brg1*-mutant brains could cause the stenosis of aqueduct and hydrocephalus.

Discussion

In this report, we demonstrated that *Camk2a-Cre*-mediated *Brg1* deletion led to perinatal hydrocephalus. Using a new reporter, we demonstrated that the *Camk2a-Cre* was unexpectedly active beginning in late embryonic stages. *Brg1* deletion in a subset of developing forebrain neurons caused abnormal development of SCO and Sylvian aqueduct in a non-cell autonomous manner, which led to the hydrocephalus defects.

Camk2a-Cre (line 93) was previously reported to be most active postnatally [3]. There is little activity before birth. Using a more sensitive reporter, we showed *Camk2a-Cre* activities begin at late embryonic stage and *Camk2a-Cre* mediated *Brg1* deletion led to perinatal hydrocephalus. Notably, another *Camk2a-Cre* line also has activity during embryonic stages [21]. The hydrocephalus phenotype is unique to the *Camk2a-Cre Brg1^{F/F}* mice and caused by *Camk2a*-mediated *Brg1* deletion since mice of the parental strains (*Camk2a-Cre Brg1^{F/+}* mice and *Brg1^{F/F}* mice) had normal brain structures. Deleting *Brg1* using other pan-neuron-specific Cre systems at similar developmental stages did not cause hydrocephalus either (our unpublished data), suggesting that the deletion of *Brg1* in specific set of neurons in combination with the wild-type surrounding brain tissues may be the cause of hydrocephalus.

Camk2a-Cre Brg1^{F/F} mice have very thin cortices, which is likely due to the damaging effect of hydrocephalus. Although Nestin-Cre-mediated *Brg1* deletion in neural progenitors also caused thinning of the cortex due to impaired progenitor proliferation starting from E12.5 [13], the thin cortices in *Camk2a-Cre Brg1^{F/F}* mice are probably not caused entirely by neural progenitor defects. *Camk2a-Cre* was only transiently expressed in a small number

of neural progenitors and thus is not likely to be responsible for the dramatic phenotypes observed in forebrain morphology at P0. Our data indicate that Camk2a-Cre-mediated *Brg1* deletion in forebrain neurons caused abnormal structures of SCO and aqueduct in a non-cell-autonomous manner, which led to hydrocephalus and consequently damaged forebrains. However, the molecular link between *Brg1* deletion and hydrocephalus is not clear. Our studies with other neuronal deletion of *Brg1* have identified several growth factors regulated by *Brg1* deletion, which may affect the growth and survival of aqueduct ependymal cells or neural cells in the surrounding regions and cause hydrocephalus. Hydrocephalus is associated with intellectual disabilities and autism. Therefore, our study suggests that deficiencies in *Brg1* activity underlie autism-related brain defects such as hydrocephalus.

Conclusions

This study demonstrated that Camk2a-Cre-mediated *Brg1* deletion led to perinatal hydrocephalus. We showed that the Camk2a-Cre (line 93) was unexpectedly active beginning in late embryonic stages. *Brg1* deletion in a subset of developing forebrain neurons caused abnormal development of SCO and Sylvian aqueduct in a non-cell-autonomous manner, which led to the hydrocephalus defects.

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Figure 1. Camk2a-Cre-mediated *Brg1* deletion causes perinatal hydrocephalus

(A) At P11, a *Camk2a-Cre Brg1^{F/F}* mouse displays a typical dome-shaped cranium (arrow) indicating hydrocephalus. A control *Brg1^{F/F}* mouse is shown on the left. (B) Representative photographs of the brains from P26 *Camk2a-Cre Brg1^{F/F}* and control *Brg1^{F/F}* mice. *Brg1*-mutant brains have enlarged hemispheres, compressed olfactory bulbs (OB), and thinner cerebral cortices compared to those of control littermates. (C) Representative H&E-stained comparable coronal brain sections of *Camk2a-Cre Brg1^{F/F}* and control *Brg1^{F/F}* mice at P0 and P11 show expanded lateral ventricles, remarkably thin cerebral cortex, and significantly reduced corpus callosum in the mutant brains. Scale bars: 500 μ m. HC: hippocampus, LV: lateral ventricle, CC: corpus callosum.

Figure 2. Camk2a-Cre is active in the forebrain early during development

(A) In PCR genotyping, only samples from *Camk2a-Cre* transgenic mice but not *Emx1-Cre* control mice produce bands from the *Camk2a*-specific primers. Both *Emx1-Cre* and *Camk2a-Cre* transgenes yield bands from primers designed to detect *Cre*. (B) Brain sections of *Camk2a-CreAi9* reporter mice at different developmental stages were stained with anti-RFP antibodies to visualize Cre-induced tdTomato expression. *Camk2a-Cre* activities in the forebrain are detected as early as E17.5. tdTomato signals in somas are largely absent from mid-hind brain regions. Scale bars: 500 μ m. (C) Brg1 staining of *Camk2a-Cre Brg1^{F/F}*

forebrain sections shows progressive Brg1 deletion from a subset of neurons in the cortex and hippocampus. Scale bars: 50 μ m. CTX: cortex, DG: dentate gyrus.

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Figure 3. Effects of Brg1 deletion on SCO and aqueduct structures are non-cell-autonomous (A) H&E staining of P11 *Camk2a-Cre Brg1^{F/F}* and littermate control *Brg1^{F/F}* midbrain sections shows that the subcommissural organ (SCO) and Sylvian aqueduct (Aq) in *Brg1*-mutant mice are smaller than those in controls. Scale bars: 50 μm . (B) Brg1 staining indicates that Brg1 is not deleted in SCO cells, the ependymal cells lining the aqueduct, or in the neural cells in the surrounding tissues in *Camk2a-Cre Brg1^{F/F}* mice at P11. The right three panels are different color channels of the same pictures. Scale bars: 10 μm . (C) Representative images of H&E stained SCO and aqueduct in the caudal regions of *Camk2a-Cre Brg1^{F/F}* and control *Brg1^{F/F}* brains at P11. The boxes indicate the regions that were used for cell quantification. The boxed regions are shown on the right at higher magnification after DAPI staining. Scale bars: 100 μm , H&E staining; 10 μm , DAPI staining. (D) Quantification of cell densities of aqueduct ependymal cells and surrounding neural cells;

both are increased in *Brg1*-mutant mice compared to controls. Cells from 4 mice in each genotype group (5 sections/mouse) were counted; representative regions are boxed in panel C. Student's t-test, **: $p < 0.01$.

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