Experimental Neurology 254 (2014) 29–40

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Conditional N-WASP knockout in mouse brain implicates actin cytoskeleton regulation in hydrocephalus pathology

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ARTICLE INFO

Article history:
Received 22 November 2013
Revised 8 January 2014
Accepted 14 January 2014
Available online 23 January 2014

Keywords:
Actin cytoskeleton
Hydrocephalus
N-WASP
Cilia
Astrogliosis
Cerebral ventricles

ABSTRACT

Cerebrospinal fluid (CSF) is produced by the choroid plexus and moved by multi-ciliated ependymal cells through the ventricular system of the vertebrate brain. Defects in the ependymal layer functionality are a common cause of hydrocephalus. N-WASP (Neural-Wiskott Aldrich Syndrome Protein) is a brain-enriched regulator of actin cytoskeleton and N-WASP knockout caused embryonic lethality in mice with neural tube and cardiac abnormalities. To shed light on the role of N-WASP in mouse brain development, we generated N-WASP conditional knockout mouse model N-WASP$^{fl/fl}$; Nestin-Cre (NKO-Nes). NKO-Nes mice were born with Mendelian ratios but exhibited reduced growth characteristics compared to their littermates containing functional N-WASP alleles. Importantly, all NKO-Nes mice developed cranial deformities due to excessive CSF accumulation and did not survive past weaning. Coronal brain sections of these animals revealed dilated lateral ventricles, defects in ciliogenesis, loss of ependymal layer integrity, reduced thickness of cerebral cortex and aqueductal stenosis. Immunostaining for N-cadherin suggests that ependymal integrity in NKO-Nes mice is lost as compared to normal morphology in the wild-type controls. Moreover, scanning electron microscopy and immunofluorescence analyses of coronal brain sections with anti-acetylated tubulin antibodies revealed the absence of cilia in ventricular walls of NKO-Nes mice indicative of ciliogenesis defects. N-WASP deficiency does not lead to altered expression of N-WASP regulatory proteins, Fyn and Cdc42, which have been previously implicated in hydrocephalus pathology. Taken together, our results suggest that N-WASP plays a critical role in normal brain development and implicate actin cytoskeleton regulation as a vulnerable axis frequently deregulated in hydrocephalus.

Introduction

In the vertebrates, the ventricular system comprises two symmetrical lateral, the third and the fourth ventricles connected with the central canal of the spinal cord (Lowery and Sive, 2009). These compartments are filled with cerebrospinal fluid (CSF) and lined by a single ependymal layer composed by multi-ciliated polarized epithelial tx1xncells (Mirzadeh et al., 2010). The beating of cilia moves the CSF through the ventricular system from the lateral ventricles to the third ventricle and through an aqueduct to the fourth ventricle (O'Callaghan et al., 2012). The CSF subsequently enters the central canal and is ultimately drained away into the circulatory system (Abouhamed et al., 2009).

Hydrocephalus, also called "water on the brain", is an abnormal medical condition caused by accumulation of the CSF in the ventricles due to a blockage of the CSF outflow or as a result of excessive CSF production (Oreskovic and Klarica, 2011). Mutations in ciliary components that affect the generation or beating of cilia cause ventricular enlargement and hydrocephalus (Banizs et al., 2005; Ibanez-Tallon et al., 2004; Lechtreck et al., 2008; Sapiro et al., 2002). Hydrocephalus resulting from an obstruction along one or more of the narrow passages connecting the ventricles is classified as non-communicating hydrocephalus, whereas hydrocephalus due to impaired absorption of the CSF in the subarachnoid space is termed as communicating hydrocephalus (Perez-Figares et al., 2001).

Several earlier studies have examined the molecular bases of hydrocephalus using genetically modified animals. For example, loss of proteins affecting epithelial cell adhesion and polarity such as Lgl1 (Klezovitch et al., 2004) and myosin IIB (Ma et al., 2007) was implicated in inducing hydrocephalus by affecting the integrity of the ependymal layer. More recently, knockout of proto-oncogenic tyrosine kinase Fyn (Fgr/Yes related Novel) has been shown to result in severe hydrocephalus at juvenile stages and premature death (Goto et al., 2008). Similarly, conditional inactivation of the small Ras-related GTPase Cdc42, an important regulator of actin cytoskeleton dynamics and cell polarity in neuroepithelial cells, caused hydrocephalus in mice due to disruption of ependymal cell differentiation and resultant aqueductal stenosis (Peng et al., 2013). However, whether these diverse factors might function through a common molecular pathway has not been investigated.

The dynamic actin cytoskeleton plays an essential role in the development and homeostasis of multicellular organism (Pula and Krause, 2008). The actin cytoskeleton made of polymerized G-actin molecules and actin

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0014-4886/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.expneurol.2014.01.011

associated proteins regulate cell shape changes, cell–extracellular matrix (ECM) interactions, cell–cell adhesion, cell migration and cell proliferation (Abouhamed et al., 2009). Although G-actin molecules can self-assemble to form F-actin in vitro, the formation and maintenance of F-actin molecules in vivo is a highly coordinated process that depends on several specialized regulatory factors (Pollard et al., 2001). N-WASP, a ubiquitous protein expressed at especially high levels in the nervous system, provides an important example of this functional category (Takenawa and Suetsugu, 2007). N-WASP regulates the actin cytoskeleton through the activation of the Arp2/3 complex consisting of Arp2, Arp3 and five additional protein components (Rohatgi et al., 1999). This function is mediated by the N-WASP C-terminal VCA domain that interacts with both Arp2 and Arp3 subunits of the Arp2/3 complex and brings these two molecules together (Egile et al., 1999).

N-WASP exists in two conformations; a closed inactive conformation formed through the interaction of the VCA domain and the basic region in which the VCA domain is restricted and unable to activate the Arp2/3 complex (Kim et al., 2000). Importantly, the switch from inactivate to active state is mediated by binding of active Cdc42 to the N-WASP GTPase-binding domain (GBD). Moreover, the open N-WASP conformation can be stabilized by phosphorylation of the Tyr253 residue (mouse; Tyr256 in human) by tyrosine kinases including Fyn (Dovas and Cox, 2010). N-WASP has been additionally shown to be critical for cell–cell adhesion (Kovacs et al., 2011a) and function cooperatively with Tuba, a Cdc42 GEF in epithelial luminogenesis (Kovacs et al., 2011a,b).

These considerations prompted us to examine the role of N-WASP in brain development by generating a conditional allele where critical *N-WASP* exons were flanked by *loxP* sites (floxed). We deleted N-WASP gene in neuroepithelial by crossing N-WASP$^{fl/fl}$ mice with Nestin-cre mice. The mice N-WASP$^{fl/fl}$ mice; nestin-cre (NKO-Nes) were born with expected Mendelian ratios and were indistinguishable from control mice (N-WASP$^{fl/WT}$; Nes-Cre) at birth. However NKO-Nes mice developed enlarged cranium, showed weight loss compared to the control mice and did not survive past the weaning age. Detailed analysis of the knockout animals suggested that the enlarged cranium was a result of hydrocephalus caused by aqueductal stenosis. These data implicate N-WASP as a critical downstream factor essential for proper development and function of the ependymal epithelium and potentially contributing to hydrocephalus pathogenesis.

Materials and method

Animals

N-WASP targeting vector was constructed by Vega Biolab (Philadelphia, PA). Targeting vector was electroporated into ES cells, and clones with targeted allele and normal karyotype were injected into blastocysts by the Yale Animal Facility for generation of chimeric mice. Germline transmission was verified before crossing with Actin-flpe mice (Rodriguez et al., 2000) to remove the Neomycin cassette to generate *N-WASP*$^{fx/WT}$ heterozygous mice. The heterozygous mice were crossed to generate *N-WASP*$^{fl/WT}$. Generation of brain specific N-WASP conditional knockout mice was carried out by mating *N-WASP*$^{fl/fl}$ animals with *Nestin-Cre* mice (Isaka et al., 1999). 25% of the pups born from this cross were expected to have the genotype, *N-WASP*$^{fl/fl}$; *Nestin-Cre*. Mice were maintained on a standard chow diet at a constant temperature of 20 °C under 12 hour/12 hour artificial light/dark cycle with unlimited access to water. All experiments were conducted according to the approved protocols of the Institutional Animal Care and Use Committee (IACUC; NTU, Singapore).

Mouse genotyping

Mouse tail tips (2–5 mm) were digested in 100 μL of Tail Digestion Buffer (TLD) containing: 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, and 0.4 mg/mL Proteinase K (Sigma-Aldrich; P2308). The tissues were incubated at 60 °C for 3 h, with gentle mixing every 30 min. Lysates were then heated at 94 °C for 10 min to denature Proteinase K. The lysates were then cleared by centrifugation at maximum speed for 15 min. Tail PCR was performed using a KAPA HiFi PCR Kit from KAPA Biosystem (KK2101). The flox/flox was detected using the primers; 5′-AGCTCAGAGAAGGTGTATTGG-3′ (forward) and 5′-AGGACTTACATCTCCAGCAAAGG-3′ (reverse). The *cre* transgene was detected using the primers; 5′-CGATGCAACGAGTGATGAGG-3′ (forward) and 5′-TCCAGGGCGCGAGTTGATAG-3′ (reverse).

Histology

Mice (P14–P18) were deeply anesthetized and perfused with 4% paraformaldehyde in PBS. Brains were fixed in 4% paraformaldehyde solution overnight at 4 °C, equilibrated in 30% sucrose solution in PBS for 24 h at 4 °C and then embedded in OCT freezing compound (Tissue-Tek) and frozen at −80 °C for further use. Coronal sections of 25 μm were obtained using a freezing microtome and sections were mounted on Superfrost slides (Fisher). For hematoxylin and eosin (H&E) staining, tissue sections were air-dried for 30 min at room temperature then dehydrated in 50% EtOH, 70% EtOH, 95% EtOH and 100% EtOH for 30 s each. The tissue sections were then stained in hematoxylin for 2 min, rinsed in water for a few times and immersed in 0.5% eosin for 2.5 min. They were then dehydrated in 50% EtOH and 70% EtOH for 30 s each, and equilibrated in 95% EtOH and 100% EtOH for 1 min each and xylene for 30 s. Tissue sections were then mounted in xylene-based DPX mounting medium (Fluka).

Scanning electron microscopy

Brain sections were first fixed with 4% paraformaldehyde followed by fixing in 2.5% glutaraldehyde overnight. Brain sections were washed several times in PBS followed by dehydration in a graded series of ethanol (30, 50, 70, 90, 100%) and finally the ethanol was substituted with hexamethyldisilazane (HMDS) before drying overnight. Sections were surface coated using a gold/palladium spattering device under optimal conditions for 2 min and sections were observed using a scanning electron microscope (JSM-7600F) at 2 kV. Observations were performed at the Facility for Analysis Characterization Testing Simulation (FACTS), NTU, Singapore.

Immunohistochemistry and immunofluorescence

For immunostaining, tissue sections prepared as described above from −80 °C were first kept at −20 °C, then 4 °C and finally air dried for 30 min at room temperature. Sections were treated with 3% H_2O_2/methanol. Immunohistochemistry analyses were performed using anti-Ki-67 (Millipore; AB9260) (1:400 dilution), anti-acetylated tubulin (T7451; sigma) (1:400 dilution), and anti-N-cadherin (610920; BD) (1:400 dilution) antibodies. Horseradish Peroxidase (HRP) was detected with VECTASTAIN® ABC kit (PK-4000) and DAB substrate (Vector Laboratories; SK-4100). For immunostaining with anti-GFAP antibody (1:100 dilution) (Millipore; AB5804), fluorescence based detection was performed using secondary antibody at 1:100 dilution.

Immunoblotting

Tissue samples (brain and liver) were rapidly dissected out from deeply anesthetized mice, immediately frozen in liquid N_2 and then stored at −80 °C for further use. To prepare tissue lysates, frozen tissue samples were homogenized in lysis buffer containing 50 mM Tris–HCl, pH 7–7.5, 200 mM NaCl, 1% Triton X 100, 0.1% SDS, 0.5% sodium deoxycholate, 10% glycerol, 1 mM EDTA, 1 mM Na Orthovanadate and 1 mM PMSF. The homogenized tissue lysate was then centrifuged at top speed, 4 °C for 10 min. Tissue lysate was collected and boiled in 2× Laemmli dye for 5 min. Protein concentration was determined