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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.

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

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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. N-WASP is activated by Fyn and Cdc42; both of which have also been 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.

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

Introduction

In the vertebrates, 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 cells (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 forth 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 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 CSF in the subarachnoid space are termed communicating hydrocephalus (Perez-Figares et al., 2001).

Several earlier studies have examined 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 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 conformation; 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 laminogenesis (Kovacs et al., 2011b) .

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^{fl/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 approved protocols of the Institutional Animal Care and Use Committee (IACUC; NTU, Singapore).

Mouse genotyping

Mouse tail tips (2-5mm) 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 hours, with gentle mixing every 30 minutes. Lysates were then heated at 94°C for 10 minutes to denature Proteinase K. The lysates were then cleared by centrifugation at maximum speed for 15 minutes. Tail PCR was performed using KAPA Hifi PCR Kit from KAPA Biosystem (KK2101). The flox/flox was detected using the primers; 5'-AGCTCAGAGAAGGTGTATTGG-3' (forward), 5'-AGGACTTACATCTCCAGCAAAGG-3' (reverse). The *cre* transgene was detected using

the primers; 5'- CGATGCAACGAGTGATGAGG-3' (forward), 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 25µm were obtained using freezing microtome and sections were mounted on Superfrost slides (Fisher). For Hematoxylin and Eosin (H&E) staining, tissue sections were air-dried for 30 minutes at room temperature then dehydrated in 50% EtOH, 70% EtOH, 95% EtOH and 100% EtOH for 30 seconds each. The tissue sections were then stained in hematoxylin for 2 minutes, rinsed in water for a few times and immersed in 0.5% eosin for 2.5 minutes. They were then dehydrated in 50% EtOH, 70% EtOH for 30 seconds each, equilibrated in 95% EtOH and 100% EtOH for 1 minute each and xylene for 30 seconds. 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 gold/palladium sputtering device under optimal conditions for 2 min and sections were observed using scanning electron microscope (JSM-7600F) at 2kV.

Observations were performed at 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 minutes at room temperature. Sections were treated with 3% H₂O₂/methanol. Immunohistochemistry analyses were performed using anti-Ki-67 (Millipore; AB9260) (1:400 dilution), anti-acetylated tubulin (T7451; sigma) (1:400 dilution), 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 N₂ 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, 1mM Na Orthovanadate and 1 mM PMSF. The homogenized tissue lysate was then centrifuged at top speed, 4°C for 10 minutes. Tissue lysate was collected and boiled in 2X Laemmli dye for 5 minutes. Protein concentration was determined using Bradford assay. 30 µg of total protein samples were resolved in 10% SDS-PAGE and immunoblotted for anti-N-WASP (1:1000 dilution), anti-Cdc42 (1:100 dilution, Santa Cruz

Biotechnology), anti-Fyn (1:500 dilution, Upstate Biotechnology), anti-GFAP (1:1000 dilution, Millipore), anti-Synaptophysin (1:1000, Invitrogen), anti-MAP2 (1:1000, Covance) and anti-GAPDH (1:10,000 dilution, Ambion Life Technology) followed by secondary antibodies (1:10,000 dilution of appropriate secondary antibody conjugated with Horseradish Peroxidase (HRP) in 3-5% skim milk solution). Subsequently, bound HRP-labeled antibodies were detected by Chemiluminescent substrate (Immobilon; Millipore, MA). Densitometry of western blots was performed using ImageJ software.

Statistical analyses

All experiments performed were performed at least three times with similar results. Student's t-test was used to analyze the statistical significance, with $*P<0.05$, $**P<0.01$, $***P<0.001$.

Results

Brain-specific conditional N-WASP knockout expression leads to premature death in young mice

To elucidate the physiological role of N-WASP in brain development, we created a mouse strain in which *N-WASP* gene was flanked by two *loxP* sites (Fig S1a). For this purpose, a targeting vector containing a G418 resistance gene (*Neo*) flanked by *FRT* sites was used to create a heterozygous (N-WASP^{Neo-floxed/WT}) mouse. The *Neo* cassette was subsequently removed by crossing the N-WASP^{Neo-floxed/WT} mice with animals expressing FLPe-recombinase (Rodriguez et al., 2000). Mice containing the resultant N-WASP^{fl/WT} allele were used to generate N-WASP^{fl/fl} homozygous mice and the homozygous mouse was used to generate N-WASP^{fl/WT}; Nes-Cre mice (Fig. S1b). PCR with P1 and P2 primers give a product of 500bp from the wild type N-WASP allele and a product of 700bp from the floxed allele. N-WASP^{fl/fl} homozygous mice was crossed with N-WASP^{fl/WT}; Nes-Cre mice to obtain nervous system-specific N-WASP conditional knockout animals (N-WASP^{fl/fl}; Nes-Cre, or NKO-Nes) (Fig. S1c). The NKO-Nes pups were born with a near-Mendelian incidence (30% vs. 25% expected). Western blot analysis of whole brain lysate from NKO-Nes and their heterozygous (N-WASP^{fl/WT}; Nest-Cre, or control) littermates confirmed the expected absence of N-WASP protein in the NKO-Nes but not in the control brain lysates (Fig S1d). On the other hand, N-WASP protein was expressed at comparable levels in NKO-Nes and control animal livers (Fig. S1d) thus confirming the nervous system (NS)-restricted N-WASP knockout.

NKO-Nes and control pups were indistinguishable at birth and appeared phenotypically normal. However, the growth rates of the NKO-Nes pups were noticeably retarded by P5-P7

as compared to their control siblings (Fig. 1a) and 100% (19 out of 19) NKO-Nes pups died prior to weaning within three-four weeks after birth (P18-P24) (Fig. 1b). On the other hand, the control mice exhibited a normal life span and no obvious defects observed in external morphology, weight, reproductive vigor, or behavior. We therefore concluded that the expression of N-WASP in the NS was essential for postnatal viability.

NS- specific *N-WASP* knockout causes symptoms of severe hydrocephalus

To understand the reasons for the neonatal mortality caused by the NS-specific loss of N-WASP, we analyzed morphological differences between NKO-Nes and control pups. Strikingly, although NKO-Nes animals displayed smaller body size compared to N-WASP^{fl/WT}; Nes-cre or N-WASP^{fl/fl} (Fig. 1c-e and data not shown), their brains (including the intracranial cerebrospinal fluid (CSF) content) were noticeably larger than those in the control cohorts. NKO-Nes mice consistently developed dome-shaped skull morphology at ~P10 which became more prominent by P15 (Fig. 1d, e) (white arrow). Visual inspection of NKO-Nes brains additionally revealed a profound decrease in vascularization as compared to the control (Fig. 1f) hinting at potential cerebrovascular arteriovenous malformation. Finally, breaking meningeal layers of NKO-Nes brains typically released unusually large amounts of CSF thus suggesting that the knockout animals were affected by hydrocephalus (Fig. 1f; black arrow). This accumulation of CSF was a likely reason for the enlargement of brain hemispheres in NKO-Nes mice (Fig. 1g). Other obvious phenotypes of the NKO-Nes animals included abnormal curvature of the thoracic vertebrae (hunchback body posture), broad-base stance/gait pattern, and locomoto disturbance with lethargic pace (data not shown).

N-WASP deficiency leads to enlarged ventricles and reduced cerebral cortex

Morphological changes in NKO-Nes brains were further characterized using histological studies. H&E-stained coronal brain sections confirmed that lateral ventricles of NKO-Nes brains were visibly dilated along their entire rostrocaudal aspect (Fig. 2c-h). Based on the quantitative measured values of the ventricular space, the hydrocephalic mice have substantially enlarged ventricles in both the anterior and posterior regions of lateral ventricles ($P < 0.001$) (Fig. 2i, j). Further neuroanatomical abnormalities included a profound reduction in caudate putamen (Fig. 2c, d), cortical layers, septum, and corpus callosum (Fig. 2d) in the NKO-Nes hydrocephalic brains. Reduced thickness of cerebral cortices was manifested by both overall compression of the layered structure and disappearance or merging of some layers potentially as a result of neuronal damage (Fig 3). We also found that the spatial alignment and cortical cell orientation were also disrupted in the hydrocephalic mice (Fig. 3f, l). Strikingly, NKO-Nes but not control brains lacked discernible hippocampi (Fig 2e-g). No morphological abnormalities were detected in the anterior regions of the frontal lobe (Fig. 2a, b).

Deletion of N-WASP in the neural lineage causes ependymal defects and stenosis of the aqueduct of Sylvius

Our additional histological analyses showed major defects in the NKO-Nes ependymal layer accompanied by a severe denudation of ependymal layers lining the ventricular wall (Fig. 4 and, 5l). N-cadherin plays a critical role in cortical morphogenesis (Kadowaki et al., 2007) and N-cadherin immune-staining in NKO-Nes brain sections revealed noticeable defects in ependymal layer integrity (Fig. 4e-h). Interestingly, in hydrocephalic brain the choroid plexus

that produces CSF showed undefined border with disrupted cell distribution and alignment as compared to control in which the outline of the normal choroid plexus is marginally defined and cell alignment is spatially juxtaposed (Fig S2). Moreover, detailed examination of the aqueduct of Sylvius connecting the third and the fourth ventricle showed that its rostral segment was abnormally narrow or complete collapsed in the NKO-Nes hydrocephalic mice (Fig. 5d-f). Histological analysis of this region showed that a unique type of columnar cells was absent in the rostral aqueduct wall in NKO-Nes brains due to aqueduct atresia and stenosis. We also noticed an enlarged lumen in the caudal part of the aqueduct and dilation of the fourth ventricle (Fig. 5j, k), suggesting that the NKO-Nes hydrocephalus is likely caused by a blocked connection between the third and fourth ventricles. Based on the analysis of H&E-stained coronal sections, an abnormal multilayered stratification of the ependymal cells lining along the caudal part of the aqueduct wall as well as increased cell numbers in the periaqueductal gray regions were also found in the NKO-Nes mice as compared to the control (Fig. 5l). Of note, obstruction of the cerebral aqueduct leading to thinning of the cortical mantle as well as macrocephaly is a common cause of fatal hydrocephalus in humans (Oreskovic and Klarica, 2011).

N-WASP-deficient brains undergo astrogliosis

Earlier clinical and animal studies identified astrogliosis, an increased incidence of activated astroglial cell, as one of the characteristic pathological symptoms of hydrocephalic brain (Deren et al., 2010; Mangano et al., 1998; Miller and McAllister, 2007). To investigate whether the hydrocephalus symptoms in NKO-Nes mice could be accompanied by similar effects, we analyzed corresponding brain samples by immunoblotting with antibodies specific to neuronal markers microtubule associated protein-2 (MAP2) and synaptophysin, as well as

astrocyte marker glial fibrillary acidic protein (GFAP). This analysis detected no apparent difference in MAP2 and synaptophysin expression between the NKO-Nes and control brains. However, NKO-Nes brains expressed dramatically increased amounts of GFAP as compared to the control samples (Fig. 6 a-d). Our further immunohistology staining for GFAP showed a marked increase in astrocyte density in the cerebral cortex and surrounding areas in the NKO-Nes brains (Fig. 6 e-h). In addition, astrocytes in NKO-Nes brains tended to have distinctively larger body size and thicker processes compared to their control counterparts (Fig. 6i, j).

Ciliary defects in N-WASP-deficient brains

Ependymal cells that line the walls of the ventricular system are multi-ciliated epithelial cells and impaired cilia formation and function are often associated with hydrocephalus (Banizs et al., 2005; Lattke et al., 2012). Thus we analyzed the ependymal cilia at P14-18 in the lateral ventricles by staining for acetylated alpha tubulin a known marker for cilia (Fig. 7a, b). Compared to the control mice, the NKO-Nes mice had reduced or no cilia suggesting defects in the formation or maintenance of cilia. In order to confirm these results we carried out scanning electron microscope (SEM) imaging (Fig. 7c-f). SEM micrographs showed that the surface of ependymal cells lining the lateral ventricles in NKO-Nes were relatively smooth in comparison to control mice which had many fine projections.

Loss of N-WASP expression does not affect the expression of its upstream regulators

NS-specific inactivation of N-WASP-interacting factors, Cdc42 and Fyn has been previously reported to cause hydrocephalus and neonatal mortality (Goto et al., 2008; Peng et al., 2013) ,

similar to the corresponding phenotypes of the NKO-Nes mice. We therefore examined the expression of Fyn and Cdc42 using immunoblotting (Fig 8a-c). Quantification of the immunoblot data showed no significant difference in the expression of the two proteins between NKO-Nes and control mouse brains (Fig. 8). Similarly, the expression of another N-WASP partner, WIP, was unchanged (Fig 8a, d). Thus, the severe hydrocephalic condition observed in the NKO-Nes mice was not due to changes in the expression of N-WASP-interacting proteins.

Discussion

Hydrocephalus is a devastating condition affecting 1-3 children per 1000 live births and is the most common neurologic disorder requiring pediatric surgery (Casey et al., 1997). It is estimated that up to 40% of hydrocephalus in humans is due to genetic factors (Haverkamp et al., 1999). Loss of several mouse genes has been reported previously to induce lethal hydrocephalus (Thumkeo et al., 2011; Vogel et al., 2012). However, the functional variety of factors identified in these studies has made it difficult to deduce unifying themes in the pathogenesis of this disorder. To this end, we show that conditional inactivation of a major actin cytoskeleton regulator N-WASP in the NS induces 100% penetrant hydrocephalus in young mice. The importance of this data is underscored by similar phenotypic effects previously reported for NS-specific knockouts of known N-WASP activators Cdc42 (Peng et al., 2013) and Fyn (Goto et al., 2008) as well as other modulators of actin cytoskeleton including Pak4 (Wells and Jones, 2010) and Myosin IXa (Abouhamed et al., 2009).

Cdc42 is known to activate N-WASP by relieving its auto-inhibitory conformation (Kim et al., 2000). Importantly, conditional knockout of Cdc42 in mice expressing Nestin-Cre resulted in ventricle enlargement, cerebral cortex hypoplasia and hydrocephalus due to aqueductal stenosis (Peng et al., 2013). The similarity of this phenotype to that observed in the NKO-Nes animals suggests that a lack of Cdc42 may result in inefficient activation of N-WASP thus phenocopying the N-WASP knockout. However Cdc42-deficient mice died within 72 hours after birth (Peng et al., 2013), i.e. noticeably earlier than the NKO-Nes mice that died between P18-P24. This suggests that nervous- system-specific inactivation of Cdc42 likely leads to deregulation of additional components. In addition to N-WASP, Cdc42 has been shown to regulate actin cytoskeleton dynamics and other cellular processes through the Pak family (serine/threonine protein Kinase) (Wells and Jones, 2010). Interestingly, conditional

knockout of Pak4 using Nestin-cre also results in hydrocephalic phenotypes. Pak4 conditionally null mice are born phenotypically normal but display growth retardation and die by 4 weeks after birth with severe hydrocephalus (Tian et al., 2011).

Activated state of N-WASP is maintained by the phosphorylation of critical Tyrosine residue by Src family of Tyrosine Kinases such as Fyn (Dovas and Cox, 2010). Notably, Fyn knockout mice were born with the expected Mendelian ratio and more than 50% of the mice developed hydrocephalus by 4 weeks of age with enlarged lateral ventricles, thinner cerebral cortices and degenerating axons in the corpus callosum (Goto et al., 2008). The reduced penetrance of Fyn knockout compared to conditional N-WASP or Cdc42 knockout is probably due to the fact that other Tyrosine kinases such as Focal Adhesion Kinase (FAK) can phosphorylate N-WASP at the same critical Tyrosine residue (Wu et al., 2004).

Our histological and immunostaining analyses reveal major defects in epithelial cell polarity in NKO-Nes brains, consistent with the role of N-WASP in maintaining epithelial integrity (Kovacs et al., 2011a). Epithelial cell polarity is known to be maintained by the Cdc42/Par6/ α PKC polarity complex containing Cdc42 as a functionally critical subunit (Chen and Zhang, 2013). Cdc42 pathway has been reported to be especially critical for faithful formation of hollow lumen structure, a process additionally requiring Tuba (Cdc42 GEF) to activate Cdc42 and orientate the spindle (Jaffe et al., 2008; Kovacs et al., 2011b) . Notably, Tuba has been shown to bind to N-WASP and stimulate actin assembly (Kovacs et al., 2006; Salazar et al., 2003). Tuba is a GEF for Cdc42, a protein that is well characterized for its role on cell polarization and has been shown to activate N-WASP (Symons et al., 1996).

Ependymal cells are multi-ciliated epithelial cells and defects in formation or function of cilia has been implicated in hydrocephalus condition (Fliegeauf et al., 2007). Immunostaining with

anti-acetylated tubulin revealed reduced cilia on the ependymal cells of NKO-Nes mice and this was further confirmed by SEM micrographs suggesting that N-WASP deficiency leads to defects in the formation of motile cilia.

Although additional work will be needed to address this directly, it is tempting to speculate that the Cdc42 and Fyn deficiencies in developing brain might cause hydrocephalus as a result of inefficient activation of N-WASP. In this model, N-WASP promotes polymerization of branched actin filaments in a Tuba-, Cdc42- and Fyn-stimulated manner (Fig. 8e). Cdc42 additionally regulates the formation of unbranched actin filaments through mDia (Wells and Jones, 2010) and Pak4 (Abo et al., 1998) (Fig. 8e). We propose that defects in both branched and unbranched actin cytoskeleton dynamics compromise epithelial cell polarity and proper ependymal layer structure possibly through disrupting tight or/and adherens junctions (Kovacs et al., 2011a) . Defective ependymal structure and function may in turn lead to hydrocephalus through mechanisms discussed below.

Stenosis of the aqueduct of Sylvius, consistently detected in the NKO-Nes brains suggests a non-communicating origin of hydrocephalus in this mouse model. Interestingly this form of non-communication hydrocephalus might be distinct from that observed in conditional knockout of Rnd3, where cerebral aqueduct was blocked likely due to ependymal epithelium overgrowth caused by up-regulated Notch signaling (Lin et al., 2013). Indeed, staining of the corresponding region for proliferation marker Ki67 revealed a significant decrease in Ki67 labeling at the subventricular zone of NKO-Nes mice, compared to control mice indicating reduced cell proliferation (Fig. S3b) similar to that reported for Pak4 conditional knockout mice (Tian et al., 2011).

We note, however, that NKO-Nes mice show microanatomic defects in other brain regions including choroid plexus, a structure responsible for the CSF production (Fig. S2a-e). It is

therefore possible that the pathological effects of aqueductal stenosis in this case are further exacerbated by a changed balance between CSF production and reabsorption. Interestingly, hydrocephalus in Fyn-null mice did not appear to be associated with stenosis (Goto et al., 2008) suggesting that increased production or reduced CSF absorption could be the major contributing factors in this system.

The novel aspect of the N-WASP biology uncovered by our work expands the growing list of known functions of this functionally versatile protein. For example, non-conditional N-WASP knockout caused embryonic lethality with defects in neural tube formation (Lommel et al., 2001; Snapper et al., 2001). N-WASP is also known to play critical roles in neurite extension (Banzai et al., 2000), neuronal morphology (Pinyol et al., 2007), neurogenesis (Liebau et al., 2011), and myelination in the peripheral nervous system (Jin et al., 2011; Novak et al., 2011).

In conclusion, we show here that that N-WASP is essential for proper brain development. Together with previously published results, our data implicate epithelial cell defects caused by the loss of N-WASP and its activators and synergists as a recurring molecular trigger of hydrocephalus.

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Figure legends

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Figure 1: Reduced growth, premature death and Abnormal skull phenotype of NKO-Nes mice.

(a) Average body weight of control and NKO-Nes pups at day 7, 10 and 13 after birth. *** representing $P < 0.001$. (b) Graph representing the incidence of premature death (100% in NKO-Nes mice, 0% in control mice). Fisher's exact test $P < 0.001$

(c, d) Side view of control (heterozygous) and NKO-Nes (homozygous) mice at P15. Compared to control mice A, the NKO-Nes mice displayed a dome shaped skull B. (e) The front view of both control and NKO-Nes mice displaying the reduced body size (in length) of NKO-Nes mice. (f) Top view of the whole skull of mice at P15. Fewer blood vessels were observed across the NKO-Nes mice skull as compared to control mice skull. A large amount of CSF was drained out from NKO-Nes mice skull during brain isolation. (g) Top view of the whole brain of mice at P15. The accumulation of CSF in NKO-Nes mice skull resulted in brain swelling as global brains showed the enlarged hemispheres in NKO-Nes mice.

Figure 2: Increased ventricular size in NKO-Nes mice.

Representative low-power photomicrographs of 25 μ m-thick coronal sections of 3-week-old littermate brains obtained from control and NKO-Nes mice. These sections are stained with H & E to characterize their gross morphological changes. Histological analysis reveals enlarged lateral ventricles of NKO-Nes mice (a'-h') as compared to the control (a-h). Morphometric analysis of the total area in the anterior (i) and posterior (j) regions of the lateral ventricle show significant enlarged ventricular space in the NKO-Nes hydrocephalic mice.

Importantly, structural anomalies were observed in the caudate-putamen, cortex, septum, and corpus callosum (**c, c', d, d'**); and also developmentally absence of hippocampal formation (**e', f'**) was found in the NKO-Nes mice brain. Note no significant morphological deterioration was detected in the anterior regions of the frontal cortex (**a, a', b, b'**). Scale bar, represents 1 mm. Abbreviation: CX, cortex; PFC, prefrontal cortex; LV, lateral ventricle; 3V, 3rd ventricle; Aq, Aqueduct; CPu, caudate putamen; S, septum; CC, corpus callosum.

Figure 3: Reduced cortical thickness in NKO-mice.

H&E stained coronal sections from control and NKO-Nes mice at post-natal 15 days. Hydrocephalic brain induces reduction of cortical thickness (**a, d, g, j**) along its rostral and caudal parts. Note, the spatial alignment and relation of cortical cell orientation were disrupted and compressed by the general enlargement of the ventricle system (**c, f, i, l**) in NKO-Nes compared to control mice. Scale bar: 500µm (**a, d, g, j**); 100µm (**b, e, h, k**) and 50µm (**c, f, i, l**). Abbreviation: CX, cortex; CPu, caudate putamen; LV, lateral ventricle.

Figure 4: Loss of neuroepithelial integrity in NKO-Nes mice.

(a-d) H & E stained coronal sections of ependymal lining from control and NKO-Nes mice. In control, high-magnification photomicrographs show the intact surface layer of ciliated ependymal lining of the lumen of the lateral ventricle wall (**b**, arrows). In contrast, NKO-Nes brain induces morphological alterations of its ventricular ependymal lining as well as its spatial cell distribution and orientation in the periventricular region (**d**, arrows). Scale bar: 100µm (**a, c**) and 50µm (**b, d**). Representative photomicrographs of immunofluorescence for DAPI and N-Cadherin (**e-h**). The panels show N-Cadherin expression restricted to ependymal cells (**e**) in the control mice while the expression of N-Cadherin was detected in both the ependymal and subventricular zone cells (**g**). Scale bar: 20µm (**e-h**). LV, lateral ventricle.

Figure 5: Dilatation and occlusion of aqueduct in NKO-mice.

H&E stained coronal sections showed stenosis of the rostral part of aqueduct (**d, e, f**) and enlargement within the canal of the caudal aqueduct (**j, k, l**) in NKO-Nes, but not in the control (**a, b, c, g, h, i**). Histological analysis displayed abnormal lining of neuroepithelial cells along the lumen of the central canal in NKO-Nes. Nevertheless, in control the lumen is lined by well differentiated ependymal cells that showed distinct layers covering the aqueduct canal surface (Arrows). Scale bar: 500µm (a, d, g, j); 100µm (b, e, h, k) and 50µm (c, f, i, l). Abbreviation: PAG, periaqueductal gray; Aq, aqueduct.

Figure 6: Expression of GFAP is upregulated in NKO-Nes mice

Western blot analysis of GFAP, MAP2, Synaptophysin from whole brain lysate of 2-3 weeks old control and NKO-Nes mice, showing a remarkable increased expression of GFAP(**a-d**). Representative photomicrographs of immunofluorescence for DAPI and GFAP (**e-h, 10X Magnification**). The panels show highly-reactive GFAP expression in both the cortex and surrounding areas of the lateral ventricular regions (**f, h**), indicating astrogliosis in the hydrocephalic NKO-Nes animals. Note a typical appearance of enlarged cell body (red arrows) and thicker processes (black arrows) are shown in these highly-expressed astrocytes of NKO-Nes mice in comparison to control (**i, j; 40X Magnification**). Scale bar: 100µm (e-h) and 50µm (i, j). Abbreviation: CX, cortex; LV, lateral ventricle. *** representing $P < 0.001$

Figure 7: N-WASP deficiency leads to ependymal cilia abnormalities.

(**a, b**) P14-18 brain sections were stained with DAPI and immunofluorescence was carried out with cilia marker anti-acetylated tubulin. NKO-Nes mice show absence of cilia Scale bar:

20µm. (d-f) SEM micrographs of ependymal cells lining the lateral ventricles. The ependymal cell lining the ventricles in NKO-Nes show a lack of cilia as compared to control mice. Scale bar: 10um (c,d), 1um (e,f).

Figure 8: Expression of N-WASP binding proteins, Fyn, Cdc42 and WIP was not altered in NKO-Nes mice compared to control mice.

a) Western blot analysis of Fyn, Cdc42 and WIP in control and NKO-Nes whole brain lysate using respective antibodies. Densitometry analysis of Cdc42 b), Fyn c) and WIP d) representing their similar expression level in control and NKO-Nes whole brain lysate. e) Actin axis in the formation and function of ependymal. Activated Cdc42 regulates the formation of branched and unbranched actin filaments through N-WASP and mDia. The polymerized actin is further reorganized by the Pak (P21 Activated Kinase) and Myosin. Deficiency of Cdc42, Fyn, Pak4, Myosin IXa or N-WASP leads to hydrocephalus.

Figure S1: Generation of N-WASP/flox mice.

(a) Schematic diagram representing the target vector and subsequent generation of conditional knockout mouse. Exons 3 and 4 of the N-WASP were floxed by the insertion of two LoxP sites and Neomycin antibiotic selection marker was also included in the targeting vector. The Neomycin gene was removed by crossing the mice with Mice expressing Flippase. (b) An example of tail PCR genotyping of 1st generation mice. Heterozygous *N-WASP^{flox/WT}; Nestin-Cre* was indicated by arrow (↑). (c) Tail PCR genotyping of back crossed mice. Homozygous *N-WASP^{flox/flox}; Nestin-Cre* (NKO-Nes) was indicated by asterisk (*). (d) Western blot analysis of N-WASP expression in whole brain lysate or mice liver lysate isolated from control heterozygous mice and NKO-Nes homozygous mice.

Figure S2: Loss of choroid plexus integrity in NKO-Nes mice

The choroid plexus in hydrocephalic brain showed undefined border with disrupted cell alignment between adjacent cells as compared to control Scale bar: 500µm (a, d); 100µm (b, e) and 50µm (c, f).

Figure S3: Effect of NKO-Nes on the progenitor and post-mitotic neurons.

Representative photomicrographs of coronal sections showing the subventricular zone. Sections are 10X magnification and stained for Ki-67, widely used marker for cell proliferation. Note the Ki-67 proliferative cells are less populated in the subventricular zone of NKO-Nes as compared to control mice (Arrows). Scale bar: 100µm Abbreviation: LV, lateral ventricle.

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

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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, that 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.

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

Introduction

In the vertebrates, 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 cells (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 forth 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 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 CSF in the subarachnoid space are termed communicating hydrocephalus (Perez-Figares et al., 2001).

Several earlier studies have examined 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 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 laminogenesis (Kovacs et al., 2011a; Kovacs et al., 2011b) .

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^{fl/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 approved protocols of the Institutional Animal Care and Use Committee (IACUC; NTU, Singapore).

Mouse genotyping

Mouse tail tips (2-5mm) 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 hours, with gentle mixing every 30 minutes. Lysates were then heated at 94°C for 10 minutes to denature Proteinase K. The lysates were then cleared by centrifugation at maximum speed for 15 minutes. Tail PCR was performed using KAPA Hifi PCR Kit from KAPA Biosystem (KK2101). The flox/flox was detected using the primers; 5'-AGCTCAGAGAAGGTGTATTGG-3' (forward), 5'-AGGACTTACATCTCCAGCAAAGG-3' (reverse). The *cre* transgene was detected using

the primers; 5'- CGATGCAACGAGTGATGAGG-3' (forward), 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 25µm were obtained using freezing microtome and sections were mounted on Superfrost slides (Fisher). For Hematoxylin and Eosin (H&E) staining, tissue sections were air-dried for 30 minutes at room temperature then dehydrated in 50% EtOH, 70% EtOH, 95% EtOH and 100% EtOH for 30 seconds each. The tissue sections were then stained in hematoxylin for 2 minutes, rinsed in water for a few times and immersed in 0.5% eosin for 2.5 minutes. They were then dehydrated in 50% EtOH, 70% EtOH for 30 seconds each, equilibrated in 95% EtOH and 100% EtOH for 1 minute each and xylene for 30 seconds. 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 gold/palladium sputtering device under optimal conditions for 2 min and sections were observed using scanning electron microscope (JSM-7600F) at 2kV.

Observations were performed at 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 minutes at room temperature. Sections were treated with 3% H₂O₂/methanol. Immunohistochemistry analyses were performed using anti-Ki-67 (Millipore; AB9260) (1:400 dilution), anti-acetylated tubulin (T7451; sigma) (1:400 dilution), 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 N₂ 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, 1mM Na Orthovanadate and 1 mM PMSF. The homogenized tissue lysate was then centrifuged at top speed, 4°C for 10 minutes. Tissue lysate was collected and boiled in 2X Laemmli dye for 5 minutes. Protein concentration was determined using Bradford assay. 30 µg of total protein samples were resolved in 10% SDS-PAGE and immunoblotted for anti-N-WASP (1:1000 dilution), anti-WIP, anti-Cdc42 (1:100 dilution,

Santa Cruz Biotechnology), anti-Fyn (1:500 dilution, Upstate Biotechnology), anti-GFAP (1:1000 dilution, Millipore), anti-Synaptophysin (1:1000, Invitrogen), anti-MAP2 (1:1000, Covance) and anti-GAPDH (1:10,000 dilution, Ambion Life Technology) followed by secondary antibodies (1:10,000 dilution of appropriate secondary antibody conjugated with Horseradish Peroxidase (HRP) in 3-5% skim milk solution). Subsequently, bound HRP-labeled antibodies were detected by Chemiluminescent substrate (Immobilon; Millipore, MA). Densitometry of western blots was performed using ImageJ software.

Statistical analyses

All experiments performed were performed at least three times with similar results. Student's t-test was used to analyze the statistical significance, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Brain-specific conditional N-WASP knockout expression leads to premature death in young mice

To elucidate the physiological role of N-WASP in brain development, we created a mouse strain in which *N-WASP* gene was flanked by two *loxP* sites (Fig. S1A). For this purpose, a targeting vector containing a G418 resistance gene (*Neo*) flanked by *FRT* sites was used to create a heterozygous ($N-WASP^{Neo-floxed/WT}$) mouse. The *Neo* cassette was subsequently removed by crossing the $N-WASP^{Neo-floxed/WT}$ mice with animals expressing FLPe-recombinase (Rodriguez et al., 2000). Mice containing the resultant $N-WASP^{fl/WT}$ allele were used to generate $N-WASP^{fl/fl}$ homozygous mice and the homozygous mouse was used to generate $N-WASP^{fl/WT}; Nes-Cre$ mice (Fig. S1B). PCR with P1 and P2 primers give a product of 500bp from the wild type *N-WASP* allele and a product of 700bp from the floxed allele. $N-WASP^{fl/fl}$ homozygous mice was crossed with $N-WASP^{fl/WT}; Nes-Cre$ mice to obtain nervous system-specific *N-WASP* conditional knockout animals ($N-WASP^{fl/fl}; Nes-Cre$, or NKO-Nes) (Fig. S1C). The NKO-Nes pups were born with a near-Mendelian incidence (30% vs. 25% expected). Western blot analysis of whole brain lysate from NKO-Nes and their heterozygous ($N-WASP^{fl/WT}; Nest-Cre$, or control) littermates confirmed the expected absence of *N-WASP* protein in the NKO-Nes but not in the control brain lysates (Fig. S1D). On the other hand, *N-WASP* protein was expressed at comparable levels in NKO-Nes and control animal livers (Fig. S1D) thus confirming the nervous system (NS)-restricted *N-WASP* knockout.

NKO-Nes and control pups were indistinguishable at birth and appeared phenotypically normal. However, the growth rates of the NKO-Nes pups were noticeably retarded by P5-P7

as compared to their control siblings (Fig. 1A) and 100% (19 out of 19) NKO-Nes pups died prior to weaning within three-four weeks after birth (P18-P24) (Fig. 1B). On the other hand, the control mice exhibited a normal life span and no obvious defects observed in external morphology, weight, reproductive vigor, or behavior. We therefore concluded that the expression of N-WASP in the NS was essential for postnatal viability.

NS- specific *N-WASP* knockout causes symptoms of severe hydrocephalus

To understand the reasons for the neonatal mortality caused by the NS-specific loss of N-WASP, we analyzed morphological differences between NKO-Nes and control pups. Strikingly, although NKO-Nes animals displayed smaller body size compared to N-WASP^{fl/wt}; Nes-cre or N-WASP^{fl/fl} (Figs. 1C-E and data not shown), their brains (including the intracranial cerebrospinal fluid (CSF) content) were noticeably larger than those in the control cohorts. NKO-Nes mice consistently developed dome-shaped skull morphology at ~P10 which became more prominent by P15 (Figs. 1D-E) (white arrow). Visual inspection of NKO-Nes brains additionally revealed a profound decrease in vascularization as compared to the control (Fig. 1F) hinting at potential cerebrovascular arteriovenous malformation. Finally, breaking meningeal layers of NKO-Nes brains typically released unusually large amounts of CSF thus suggesting that the knockout animals were affected by hydrocephalus (Fig. 1F; black arrow). This accumulation of CSF was a likely reason for the enlargement of brain hemispheres in NKO-Nes mice (Fig. 1G). Other obvious phenotypes of the NKO-Nes animals included abnormal curvature of the thoracic vertebrae (hunchback body posture), broad-base stance/gait pattern, and locomoto disturbance with lethargic pace (data not shown).

N-WASP deficiency leads to enlarged ventricles and reduced cerebral cortex

Morphological changes in NKO-Nes brains were further characterized using histological studies. H&E-stained coronal brain sections confirmed that lateral ventricles of NKO-Nes brains were visibly dilated along their entire rostrocaudal aspect (Figs. 2C-H). Based on the quantitative measured values of the ventricular space, the hydrocephalic mice have substantially enlarged ventricles in both the anterior and posterior regions of lateral ventricles ($p < 0.001$) (Figs. 2I-J). Further neuroanatomical abnormalities included a profound reduction in caudate putamen (Figs. 2C-D), cortical layers, septum, and corpus callosum (Fig. 2D) in the NKO-Nes hydrocephalic brains. Reduced thickness of cerebral cortices was manifested by both overall compression of the layered structure and disappearance or merging of some layers potentially as a result of neuronal damage (Fig. 3). We also found that the spatial alignment and cortical cell orientation were also disrupted in the hydrocephalic mice (Figs. 3F, L). Strikingly, NKO-Nes but not control brains lacked discernible hippocampi (Figs. 2E-G). No morphological abnormalities were detected in the anterior regions of the frontal lobe (Figs. 2A-B).

Deletion of N-WASP in the neural lineage causes ependymal defects and stenosis of the aqueduct of Sylvius

Our additional histological analyses showed major defects in the NKO-Nes ependymal layer accompanied by a severe denudation of ependymal layers lining the ventricular wall (Figs. 4, 5L). N-cadherin plays a critical role in cortical morphogenesis (Kadowaki et al., 2007) and N-cadherin immune-staining in NKO-Nes brain sections revealed noticeable defects in

ependymal layer integrity (Figs. 4E-H). Interestingly, in hydrocephalic brain the choroid plexus that produces CSF showed undefined border with disrupted cell distribution and alignment as compared to control in which the outline of the normal choroid plexus is marginally defined and cell alignment is spatially juxtaposed (Fig. S2). Moreover, detailed examination of the aqueduct of Sylvius connecting the third and the fourth ventricle showed that its rostral segment was abnormally narrow or complete collapsed in the NKO-Nes hydrocephalic mice (Figs. 5D-F). Histological analysis of this region showed that a unique type of columnar cells was absent in the rostral aqueduct wall in NKO-Nes brains due to aqueduct atresia and stenosis. We also noticed an enlarged lumen in the caudal part of the aqueduct and dilation of the fourth ventricle (Figs. 5J-K), suggesting that the NKO-Nes hydrocephalus is likely caused by a blocked connection between the third and fourth ventricles. Based on the analysis of H&E-stained coronal sections, an abnormal multilayered stratification of the ependymal cells lining along the caudal part of the aqueduct wall as well as increased cell numbers in the periaqueductal gray regions were also found in the NKO-Nes mice as compared to the control (Fig. 5L). Of note, obstruction of the cerebral aqueduct leading to thinning of the cortical mantle as well as macrocephaly is a common cause of fatal hydrocephalus in humans (Oreskovic and Klarica, 2011).

N-WASP-deficient brains undergo astrogliosis

Earlier clinical and animal studies identified astrogliosis, an increased incidence of activated astroglial cell, as one of the characteristic pathological symptoms of hydrocephalic brain (Deren et al., 2010; Mangano et al., 1998; Miller and McAllister, 2007). To investigate whether the hydrocephalus symptoms in NKO-Nes mice could be accompanied by similar effects, we analyzed corresponding brain samples by immunoblotting with antibodies specific

to neuronal markers microtubule associated protein-2 (MAP2) and synaptophysin, as well as astrocyte marker glial fibrillary acidic protein (GFAP). This analysis detected no apparent difference in MAP2 and synaptophysin expression between the NKO-Nes and control brains. However, NKO-Nes brains expressed dramatically increased amounts of GFAP as compared to the control samples (Figs. 6A-D). Our further immunohistology staining for GFAP showed a marked increase in astrocyte density in the cerebral cortex and surrounding areas in the NKO-Nes brains (Figs. 6E-H). In addition, astrocytes in NKO-Nes brains tended to have distinctively larger body size and thicker processes compared to their control counterparts (Figs. 6I-J).

Ciliary defects in N-WASP-deficient brains

Ependymal cells that line the walls of the ventricular system are multi-ciliated epithelial cells and impaired cilia formation and function are often associated with hydrocephalus (Banizs et al., 2005; Lattke et al., 2012). Thus we analyzed the ependymal cilia at P14-18 in the lateral ventricles by staining for acetylated alpha tubulin a known marker for cilia (Figs. 7A-B). Compared to the control mice, the NKO-Nes mice had reduced or no cilia suggesting defects in the formation or maintenance of cilia. In order to confirm these results we carried out scanning electron microscope (SEM) imaging (Figs. 7C-F). SEM micrographs showed that the surface of ependymal cells lining the lateral ventricles in NKO-Nes were relatively smooth in comparison to control mice which had many fine projections.

Loss of N-WASP expression does not affect the expression of its upstream regulators

NS-specific inactivation of N-WASP-interacting factors, Cdc42 and Fyn has been previously reported to cause hydrocephalus and neonatal mortality (Goto et al., 2008; Peng et al., 2013), similar to the corresponding phenotypes of the NKO-Nes mice. We therefore examined the expression of Fyn and Cdc42 using immunoblotting (Figs. 8A-C). Quantification of the immunoblot data showed no significant difference in the expression of the two proteins between NKO-Nes and control mouse brains (Fig. 8). Similarly, the expression of another N-WASP partner, WIP, was unchanged (Figs. 8A, D). Thus, the severe hydrocephalic condition observed in the NKO-Nes mice was not due to changes in the expression of N-WASP-interacting proteins.

Discussion

Hydrocephalus is a devastating condition affecting 1-3 children per 1000 live births and is the most common neurologic disorder requiring pediatric surgery (Casey et al., 1997). It is estimated that up to 40% of hydrocephalus in humans is due to genetic factors (Haverkamp et al., 1999). Loss of several mouse genes has been reported previously to induce lethal hydrocephalus (Thumkeo et al., 2011; Vogel et al., 2012). However, the functional variety of factors identified in these studies has made it difficult to deduce unifying themes in the pathogenesis of this disorder. To this end, we show that conditional inactivation of a major actin cytoskeleton regulator N-WASP in the NS induces 100% penetrant hydrocephalus in young mice. The importance of this data is underscored by similar phenotypic effects previously reported for NS-specific knockouts of known N-WASP activators Cdc42 (Peng et al., 2013) and Fyn (Goto et al., 2008) as well as other modulators of actin cytoskeleton including Pak4 (Wells and Jones, 2010) and Myosin IXa (Abouhamed et al., 2009).

Cdc42 is known to activate N-WASP by relieving its auto-inhibitory conformation (Kim et al., 2000). Importantly, conditional knockout of Cdc42 in mice expressing Nestin-Cre resulted in ventricle enlargement, cerebral cortex hypoplasia and hydrocephalus due to aqueductal stenosis (Peng et al., 2013). The similarity of this phenotype to that observed in the NKO-Nes animals suggests that a lack of Cdc42 may result in inefficient activation of N-WASP thus phenocopying the N-WASP knockout. However Cdc42-deficient mice died within 72 hours after birth (Peng et al., 2013), i.e. noticeably earlier than the NKO-Nes mice that died between P18-P24. This suggests that nervous- system-specific inactivation of Cdc42 likely leads to deregulation of additional components. In addition to N-WASP, Cdc42 has been shown to regulate actin cytoskeleton dynamics and other cellular processes through the Pak

family (serine/threonine protein Kinase) (Wells and Jones, 2010). Interestingly, conditional knockout of Pak4 using Nestin-cre also results in hydrocephalic phenotypes. Pak4 conditionally null mice are born phenotypically normal but display growth retardation and die by 4 weeks after birth with severe hydrocephalus (Tian et al., 2011).

Activated state of N-WASP is maintained by the phosphorylation of critical Tyrosine residue by Src family of Tyrosine Kinases such as Fyn (Dovas and Cox, 2010). Notably, Fyn knockout mice were born with the expected Mendelian ratio and more than 50% of the mice developed hydrocephalus by 4 weeks of age with enlarged lateral ventricles, thinner cerebral cortices and degenerating axons in the corpus callosum (Goto et al., 2008). The reduced penetrance of Fyn knockout compared to conditional N-WASP or Cdc42 knockout is probably due to the fact that other Tyrosine kinases such as Focal Adhesion Kinase (FAK) can phosphorylate N-WASP at the same critical Tyrosine residue (Wu et al., 2004).

Our histological and immunostaining analyses reveal major defects in epithelial cell polarity in NKO-Nes brains, consistent with the role of N-WASP in maintaining epithelial integrity (Kovacs et al., 2011a). Epithelial cell polarity is known to be maintained by the Cdc42/Par6/ α PKC polarity complex containing Cdc42 as a functionally critical subunit (Chen and Zhang, 2013). Cdc42 pathway has been reported to be especially critical for faithful formation of hollow lumen structure, a process additionally requiring Tuba (Cdc42 GEF) to activate Cdc42 and orientate the spindle (Jaffe et al., 2008; Kovacs et al., 2011b). Notably, Tuba has been shown to bind to N-WASP and stimulate actin assembly (Kovacs et al., 2006; Salazar et al., 2003). Tuba is a GEF for Cdc42, a protein that is well characterized

for its role on cell polarization and has been shown to activate N-WASP (Symons et al., 1996).

Ependymal cells are multi-ciliated epithelial cells and defects in formation or function of cilia has been implicated in hydrocephalus condition (Fliegauf et al., 2007). Immunostaining with anti-acetylated tubulin revealed reduced cilia on the ependymal cells of NKO-Nes mice and this was further confirmed by SEM micrographs suggesting that N-WASP deficiency leads to defects in the formation of motile cilia.

Although additional work will be needed to address this directly, it is tempting to speculate that the Cdc42 and Fyn deficiencies in developing brain might cause hydrocephalus as a result of inefficient activation of N-WASP. In this model, N-WASP promotes polymerization of branched actin filaments in a Tuba-, Cdc42- and Fyn-stimulated manner (Fig. 8E). Cdc42 additionally regulates the formation of unbranched actin filaments through mDia (Wells and Jones, 2010) and Pak4 (Abo et al., 1998) (Fig. 8E). We propose that defects in both branched and unbranched actin cytoskeleton dynamics compromise epithelial cell polarity and proper ependymal layer structure possibly through disrupting tight or/and adherens junctions (Kovacs et al., 2011a). Defective ependymal structure and function may in turn lead to hydrocephalus through mechanisms discussed below.

Stenosis of the aqueduct of Sylvius, consistently detected in the NKO-Nes brains suggests a non-communicating origin of hydrocephalus in this mouse model. Interestingly this form of non-communication hydrocephalus might be distinct from that observed in conditional knockout of Rnd3, where cerebral aqueduct was blocked likely due to ependymal epithelium overgrowth caused by up-regulated Notch signaling (Lin et al., 2013). Indeed, staining of the corresponding region for proliferation marker Ki67 revealed a significant decrease in Ki67 labeling at the subventricular zone of NKO-Nes mice, compared to control mice indicating

reduced cell proliferation (Fig. S3B) similar to that reported for Pak4 conditional knockout mice (Tian et al., 2011).

We note, however, that NKO-Nes mice show microanatomic defects in other brain regions including choroid plexus, a structure responsible for the CSF production (Figs. S2A-E). It is therefore possible that the pathological effects of aqueductal stenosis in this case are further exacerbated by a changed balance between CSF production and reabsorption. Interestingly, hydrocephalus in Fyn-null mice did not appear to be associated with stenosis (Goto et al., 2008) suggesting that increased production or reduced CSF absorption could be the major contributing factors in this system.

The novel aspect of the N-WASP biology uncovered by our work expands the growing list of known functions of this functionally versatile protein. For example, non-conditional N-WASP knockout caused embryonic lethality with defects in neural tube formation (Lommel et al., 2001; Snapper et al., 2001). N-WASP is also known to play critical roles in neurite extension (Banzai et al., 2000), neuronal morphology (Pinyol et al., 2007), neurogenesis (Liebau et al., 2011), and myelination in the peripheral nervous system (Jin et al., 2011; Novak et al., 2011).

In conclusion, we show here that that N-WASP is essential for proper brain development. Together with previously published results, our data implicate epithelial cell defects caused by the loss of N-WASP and its activators and synergists as a recurring molecular trigger of hydrocephalus.

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Figure legends

Figure legends

Fig. 1. Reduced growth, premature death and abnormal skull phenotype of NKO-Nes mice.

(A) Average body weight of control and NKO-Nes pups at day 7, 10 and 13 after birth. *** representing $p < 0.001$. (B) Graph representing the incidence of premature death (100% in NKO-Nes mice, 0% in control mice). Fisher's exact test $p < 0.001$

(C, D) Side view of control (heterozygous) and NKO-Nes (homozygous) mice at P15. Compared to control mice C, the NKO-Nes mice displayed a dome shaped skull D. (E) The front view of both control and NKO-Nes mice displaying the reduced body size (in length) of NKO-Nes mice. (F) Top view of the whole skull of mice at P15. Fewer blood vessels were observed across the NKO-Nes mice skull as compared to control mice skull. A large amount of CSF was drained out from NKO-Nes mice skull during brain isolation. (G) Top view of the whole brain of mice at P15. The accumulation of CSF in NKO-Nes mice skull resulted in brain swelling as global brains showed the enlarged hemispheres in NKO-Nes mice.

Fig. 2. Increased ventricular size in NKO-Nes mice.

Representative low-power photomicrographs of 25 μm -thick coronal sections of 3-week-old littermate brains obtained from control and NKO-Nes mice. These sections are stained with H & E to characterize their gross morphological changes. Histological analysis reveals enlarged lateral ventricles of NKO-Nes mice (C'-H') as compared to the control (C-H). Morphometric analysis of the total area in the anterior (I) and posterior (J) regions of the lateral ventricle show significant enlarged ventricular space in the NKO-Nes hydrocephalic mice. ***

representing $p < 0.001$. Importantly, structural anomalies were observed in the caudate-putamen, cortex, septum, and corpus callosum (C, C', D, D'); and also developmentally absence of hippocampal formation (E', F') was found in the NKO-Nes mice brain. Note no significant morphological deterioration was detected in the anterior regions of the frontal cortex (A, A', B, B'). Scale bar, represents 1 mm. Abbreviation: CX, cortex; PFC, prefrontal cortex; LV, lateral ventricle; 3V, 3rd ventricle; Aq, Aqueduct; CPu, caudate putamen; S, septum; CC, corpus callosum.

Fig. 3. Reduced cortical thickness in NKO-mice.

H&E stained coronal sections from control and NKO-Nes mice at post-natal 15 days. Hydrocephalic brain induces reduction of cortical thickness (A, D, G, J) along its rostral and caudal parts. Note, the spatial alignment and relation of cortical cell orientation were disrupted and compressed by the general enlargement of the ventricle system (C, F, I, L) in NKO-Nes compared to control mice. Scale bar: 500 μ m (A, D, G, J); 100 μ m (B, E, H, K) and 50 μ m (C, F, I, L). Abbreviation: CX, cortex; CPu, caudate putamen; LV, lateral ventricle.

Fig. 4. Loss of neuroepithelial integrity in NKO-Nes mice.

(A-D) H & E stained coronal sections of ependymal lining from control and NKO-Nes mice. In control, high-magnification photomicrographs show the intact surface layer of ciliated ependymal lining of the lumen of the lateral ventricle wall (B, arrows). In contrast, NKO-Nes brain induces morphological alterations of its ventricular ependymal lining as well as its spatial cell distribution and orientation in the periventricular region (D, arrows). Scale bar: 100 μ m (A, C) and 50 μ m (B, D). Representative photomicrographs of immunofluorescence for DAPI and N-Cadherin (E-H). The panels show N-Cadherin expression restricted to ependymal cells (E) in the control mice while the expression of N-Cadherin was detected in

both the ependymal and subventricular zone cells (**G**). Scale bar: 20 μ m (E-H). LV, lateral ventricle.

Fig. 5. Dilatation and occlusion of aqueduct in NKO-mice.

H&E stained coronal sections showed stenosis of the rostral part of aqueduct (D, E, F) and enlargement within the canal of the caudal aqueduct (J, K, L) in NKO-Nes, but not in the control (A, B, C, G, H, I). Histological analysis displayed abnormal lining of neuroepithelial cells along the lumen of the central canal in NKO-Nes. Nevertheless, in control the lumen is lined by well differentiated ependymal cells that showed distinct layers covering the aqueduct canal surface (Arrows). Scale bar: 500 μ m (A, D, G, J); 100 μ m (B, E, H, K) and 50 μ m (C, F, I, L). Abbreviation: PAG, periaqueductal gray; Aq, aqueduct.

Fig. 6. Expression of GFAP is upregulated in NKO-Nes mice

Western blot analysis of GFAP, MAP2, Synaptophysin from whole brain lysate of 2-3 weeks old control and NKO-Nes mice, showing a remarkable increased expression of GFAP (A-D). Representative photomicrographs of immunofluorescence for DAPI and GFAP (E-H, 10X Magnification). The panels show highly-reactive GFAP expression in both the cortex and surrounding areas of the lateral ventricular regions (F, H), indicating astrogliosis in the hydrocephalic NKO-Nes animals. Note a typical appearance of enlarged cell body (red arrows) and thicker processes (black arrows) are shown in these highly-expressed astrocytes of NKO-Nes mice in comparison to control (I, J; 40X Magnification). Scale bar: 100 μ m (E-H) and 50 μ m (I, J). Abbreviation: CX, cortex; LV, lateral ventricle. *** representing $p < 0.001$

Fig. 7. N-WASP deficiency leads to ependymal cilia abnormalities.

(A, B) P14-18 brain sections were stained with DAPI and immunofluorescence was carried out with cilia marker anti-acetylated tubulin. NKO-Nes mice show absence of cilia Scale bar: 20µm. (D-F) SEM micrographs of ependymal cells lining the lateral ventricles. The ependymal cell lining the ventricles in NKO-Nes show a lack of cilia as compared to control mice. Scale bar: 10µm (C, D), 1µm (E, F).

Fig. 8. Expression of N-WASP binding proteins, Fyn, Cdc42 and WIP was not altered in NKO-Nes mice compared to control mice.

(A) Western blot analysis of Fyn, Cdc42 and WIP in control and NKO-Nes whole brain lysate using respective antibodies. Densitometry analysis of Cdc42 (B), Fyn (C), and WIP (D) representing their similar expression level in control and NKO-Nes whole brain lysate. (E) Actin axis in the formation and function of ependymal. Activated Cdc42 regulates the formation of branched and unbranched actin filaments through N-WASP and mDia. The polymerized actin is further reorganized by the Pak (P21 Activated Kinase) and Myosin. Deficiency of Cdc42, Fyn, Pak4, Myosin IXa or N-WASP leads to hydrocephalus.

Fig. S1. Generation of N-WASP/flox mice.

(A) Schematic diagram representing the target vector and subsequent generation of conditional knockout mouse. Exons 3 and 4 of the N-WASP were floxed by the insertion of two LoxP sites and Neomycin antibiotic selection marker was also included in the targeting vector. The Neomycin gene was removed by crossing the mice with Mice expressing Flippase. (B) An example of tail PCR genotyping of 1st generation mice. Heterozygous *N-WASP^{flox/WT}; Nestin-Cre* was indicated by arrow (↑). (C) Tail PCR genotyping of back crossed mice. Homozygous *N-WASP^{flox/flox}; Nestin-Cre* (NKO-Nes) was indicated by asterisk (*). (D) Western blot analysis of N-WASP expression in whole brain lysate or mice liver lysate isolated from control heterozygous mice and NKO-Nes homozygous mice.

Fig. S2. Loss of choroid plexus integrity in NKO-Nes mice

The choroid plexus in hydrocephalic brain (NKO-Nes) showed undefined border with disrupted cell alignment between adjacent cells as compared to control mice brain. Scale bar: 500µm (A, D); 100µm (B, E) and 50µm (C, F).

Fig. S3. Effect of NKO-Nes on the progenitor and post-mitotic neurons.

Representative photomicrographs of coronal sections showing the subventricular zone. Sections are 10X magnification and stained for Ki-67, widely used marker for cell proliferation. Note the Ki-67 proliferative cells are less populated in the subventricular zone of NKO-Nes as compared to control mice (Arrows). Scale bar: 100µm Abbreviation: LV, lateral ventricle.

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Fig 1

Figure2
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Fig 2

Fig 3

Figure4
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Fig 5

Figure6

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Fig 6

A

B

C

D

Cortex

Peri-ventricular

Figure7
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Fig 7

Fig 8

Supplementary Material

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