Physiological Significance of *WDR45*, A Responsible Gene for β -propeller Protein Associated Neurodegeneration (BPAN), in Brain Development

Mariko Noda

Institute for Developmental Research, Aichi Developmental Disability Center

Hidenori Ito

Institute for Developmental Research, Aichi Developmental Disability Center

Koh-ichi Nagata (✉ knagata@inst-hsc.jp)

Nagoya University

Research Article

Keywords: WDR45, corticogenesis, dendrite arborization, axon pathfinding, spine formation

Posted Date: December 30th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-132972/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Scientific Reports on November 19th, 2021. See the published version at <https://doi.org/10.1038/s41598-021-02123-3>.

Abstract

WDR45 plays an essential role in the early stage of autophagy. *De novo* heterozygous mutations in *WDR45* have been known to cause b-propeller protein-associated neurodegeneration (BPAN), a subtype of neurodegeneration with brain iron accumulation (NBIA). Although BPAN patients display global developmental delay including intellectual disability, neurodevelopmental pathophysiology of BPAN remains largely unknown. In the present study, we analyzed the physiological role of Wdr45 and pathophysiological significance of the gene abnormality during mouse brain development. Morphological and biochemical analyses revealed that Wdr45 is expressed in a developmental stage-dependent manner in mouse brain. Wdr45 was also found to be located in the excitatory synapses in biochemical fractionation. Since the *WDR45* mutations are thought to cause protein degradation, we conducted acute knockdown experiments by an *in utero* electroporation method with mice to recapitulate the pathophysiological conditions of BPAN. Silencing of *Wdr45* caused abnormal dendritic development and synaptogenesis during corticogenesis, both of which were significantly rescued by co-expression with RNAi-resistant version of Wdr45. In addition, terminal arbors of callosal axons were less developed in Wdr45-deficient cortical neurons of adult mouse when compared to the control cells. These results strongly suggest a pathophysiological significance of *WDR45* gene abnormalities in neurodevelopmental aspects of BPAN.

Introduction

Autophagy is a highly dynamic and complex process essential for cell growth, survival, development and death ^{1,2}. Autophagy involves a sequential set of events including autophagosome formation, maturation, fusion with lysosomes, subsequent breakdown and the release of macromolecules back into the cytosol ². WDR45 (also known as WIPI4), an evolutionary conserved b-propeller scaffold protein, is a component of the autophagy machinery and controls an early step of autophagosome formation ³.

De novo heterozygous mutations in *WDR45* have recently been shown to cause b-propeller protein-associated neurodegeneration (BPAN), also known as static encephalopathy of childhood with neurodegeneration in adulthood (SENDA) ⁴, which is a subtype of neurodegeneration with brain iron accumulation (NBIA) ⁵. Typical presentation of BPAN consists of global developmental delay in early childhood with intellectual disability (ID), with or without concurrent spasticity, and then the patients develop sudden onset of progressive dystonia, parkinsonism and dementia during their 20s to early 30s. In the course of clinical deterioration, iron accumulation becomes evident in the substantia nigra and globus pallidus based on brain imaging ⁶. Since WDR45 has a crucial role in autophagy, an impaired autophagy mechanism has been considered to underlie the etiology of BPAN. Indeed, the autophagic flux, the whole process of autophagy, has been reported to be reduced in lymphoblastoid cell lines (LCLs) from BPAN patients ^{4,7}. Also, WDR45 protein expression was severely reduced in LCLs derived from all BPAN patients analyzed, strongly suggesting that *WDR45* mutations make the protein unstable and undergo degradation ⁴, and finally disturb the autophagosome formation and subsequent processes. Interestingly,

mutations in *WDR45* have genetic overlaps with those from neurodevelopmental disorder patients with ID and epileptic encephalopathy ⁸, indicative of an essential role of WDR45 during brain development. Loss-of-function of WDR45 is thus most likely to contribute to pathogenesis of not only BPAN but also neurodevelopmental disorders.

In this study, we tried to clarify the pathophysiological significance of *WDR45* gene abnormalities in neurodevelopmental delay, especially ID, observed in infancy of BPAN patients. To this end, we first performed expression analyses and found that Wdr45 is expressed in mouse brain in a developmental stage-dependent manner. Subsequent functional analyses revealed essential roles of Wdr45 in dendrite development, axon pathfinding and synapse formation. These results indicate physiological relevance of WDR45 during brain development and involvement of its functional deficiency in the clinical features of neurodevelopmental aspects of BPAN.

Results

Expression profile of Wdr45 protein in mouse tissues

When the expression profile was analyzed in a variety of mouse tissues by western blotting, Wdr45 with a molecular mass of ~40 kDa was detected in all tissues except small intestine (Fig.1A). While this ~40 kDa band is a major molecule in the brain tissues, liver, spleen, pancreas, ovary and uterus, it was weakly detected in heart, adrenal gland and skeletal muscle. An ~50 kDa band was detected in brain, heart and skeletal muscle, and an ~85 kDa protein was relatively well expressed in liver and kidney. Meanwhile, an ~110 kDa band was strongly and moderately expressed in pancreas and liver, respectively. In addition, tissue-specific bands with ~60 kDa, ~70 kDa and ~28 kDa were observed in the brain tissues, pancreas and skeletal muscle, respectively.

To gain some insight into the involvement of Wdr45 in neuronal development, we looked into its expression level in the whole brain extracts prepared from various developmental stages (Fig. 1B). Wdr45 with ~40 kDa was strongly detected during embryonic stages and then gradually decreased after postnatal day (P)8 to the lowest level at P30 (Fig. 1B). The ~110 kDa band also showed an expression pattern similar to that of the ~40 kDa protein, whereas weak expression of the ~50 kDa and ~60 kDa bands appeared to be weakly visualized after birth.

Although the expression level was not high in adult brain, Wdr45 is likely to be enriched at specific intracellular compartment such as synapses from the fact that functional defects in WDR45 is responsible for neurodevelopmental defects in BPAN. Biochemical fractionation of adult mouse brain was thus conducted, and the quality of each fraction was confirmed by detection of synaptophysin and PSD95, markers for pre- and post-synapses, respectively (Fig. 1C). Consequently, the ~40 kDa and ~80 kDa proteins, but not the ~50 kDa one, were detected in highly purified post synaptic density (PSD) (PSD-II) fraction where synaptophysin was little detected (Fig. 1C). The ~110 kDa and ~50 kDa proteins were only faintly observed at the PSD fraction (Fig. 1C). These results suggest that certain Wdr45 isoforms are

distributed at PSD. Given that the synaptosome fraction contains both pre- and post-synaptic components and PSD-I fraction contains contaminated presynaptic proteins, Wdr45 also might be present in the pre-synaptic compartment. Taken together, we assume that Wdr45 is involved in synapse structure and/or function in differentiated neurons.

Immunohistochemical analysis of WDR45 in mouse brain

To determine the localization of Wdr45, we performed immunohistochemical analyses of cerebral cortex at embryonic day (E) 15, E17, P0, P7 and P30. At E15 and E17, Wdr45 was broadly detected in neurons in the marginal zone (MZ), cortical plate (CP), subplate (SP) and intermediate zone (IMZ) as well as neuronal progenitors in the ventricular zone (VZ) and subventricular zone (SVZ) (Fig. 2A, a and b, B). Although the broad distribution pattern was observed until P30, subcellular distribution altered after P7; relative nuclear enrichment of Wdr45 was frequently observed from E15 to P7 while cytoplasmic distribution came to be obvious at P30 (Fig. 2A,e, C). Meanwhile, it is noteworthy that Wdr45 was detected at the ventricular surface of neural progenitors from E15 to P0 (Fig. 2B). In addition, distinct immunoreactivity was observed in the neuropil of cerebral cortex at P30 (Fig. 2D), with a strong suggestion of enrichment at excitatory synapses. Collectively, Wdr45 was expressed in the nucleus of cortical neurons and the ventricular surface during corticogenesis and its subcellular localization changed from the nucleus to the cytoplasm and then neuropils in developed cerebral cortex.

We then carried out immunohistochemical analyses in hippocampus at E17, P0, P7 and P30 and in cerebellum at P30 (Supplementary Fig. 1). As in the case of cerebral cortex, Wdr45 appeared to be expressed in the nucleus and cytoplasm at E17 and P0 in the CA regions and dentate gyrus of hippocampus (Supplementary Fig. 1A and B). Then, the cytoplasmic distribution in the neurons of CA regions as well as dentate gyrus came to be detected at P7 and became clearer at P30 (Supplementary Fig. 1C and D). In cerebellum, almost all regions were positive for Wdr45, with relatively strong expression in Purkinje cells (Supplementary Fig. 1E). These results indicate possible roles of Wdr45 in hippocampus and cerebellum.

Characterization of RNAi- and expression-vectors

Given that *WDR45* gene abnormalities are responsible for the early onset of ID in BPAN, Wdr45 is most likely to participate in the formation of cortical architecture. BPAN-causative mutants of WDR45 are supposed to be structurally unstable and undergo degradation⁴. Indeed, a BPAN-causative mutant, c.700C > T/p.(Arg234*) (Wdr45-Rstop), as well as another mutant, c.439G>T (GVmut), with single amino acid substitution in the linker region between third and fourth b-propeller domains were hardly detected when expressed in primary cultured mouse cortical neurons (Fig. 3A). We thus conducted acute knockdown experiments to recapitulate the pathophysiological conditions in BPAN. To this end, 2 RNAi vectors were designed against mouse Wdr45, pSuper-Wdr45#1 and #2, both of which effectively knocked down Myc-Wdr45 expressed in COS7 cells (Fig. 3B). In addition, pSuper-Wdr45#1 and #2 silenced

endogenous Wdr45 at least partially in cortical neurons *in vitro* (Fig. 3C). An RNAi-resistant version of Wdr45, Wdr45R, was shown to be resistant against pSuper-Wdr45#1 (Fig. 3D).

Role of Wdr45 in cortical neuron migration

We asked whether Wdr45 is involved in the migration of newly generated cortical neurons by *in utero* electroporation-based acute knockdown of Wdr45. pSuper-H1.shLuc (control), pSuper-Wdr45#1 or #2 was co-electroporated with pCAG-Turbo RFP into VZ progenitor cells, and electroporated brains were fixed and analyzed at P2. When compared to the control experiments, Wdr45-silencing had little effects on the migration and the cells were considered to normally make it to the layer II-IV (Supplementary Fig. 2).

Role of Wdr45 in axon extension and dendritic arbor development during brain development

Since aberrant synaptic network formation is tightly associated with defective brain development and function, Wdr45 may take part in axon pathfinding and/or dendrite growth during brain development. We thus first examined the interhemispheric axon projection of Wdr45-deficient cortical neurons *in vivo*. When Wdr45 was silenced in the VZ cells at E14.5, axon bundles from the hemisphere containing Wdr45-deficient cells normally reached the contralateral hemisphere at P2 (Fig. 4A) and this tendency was still observed at P30 (Fig. 4B). On the other hand, it is notable that axons of the deficient cells did not penetrate efficiently into the layer structure of contralateral cortex at P60 when compared to the control axons (Fig. 4C). Taken together, Wdr45 appeared to be important for callosal axon extension into the cortical structure after normal pathfinding from the ipsilateral to contralateral cortex.

We then examined the knockdown effects on dendritic arbor development of cortical neurons *in vivo*. Electroporation of pSuper-Wdr45#1 or #2 into the VZ cells at E14.5 gave rise to highly suppressed dendritic arborization at P10 (Fig. 5A). Sholl analyses revealed a significant decrease in the branch point number in the deficient neurons at P10 (Fig. 5B). Rescue experiments were then performed to exclude off-target effects. When an RNAi-resistant version of Wdr45, Wdr45R, was co-electroporated into the VZ progenitor cells with pSuper-Wdr45#1, morphological defects were rescued, indicating specific effects by Wdr45-silencing (Fig. 5A and B). We then measured the knockdown effects on the apical and basal dendrites, separately. As shown in Fig. 5C, apical oriented dendritic length was abnormally shortened in pSuper-Wdr45#1- or #2-transfected cells at P10, and the phenotype by pSuper-Wdr45#1 was rescued at least partially (Fig. 5C). Likewise, Wdr45-knockdown by pSuper-Wdr45#1 or #2 suppressed the basal dendrite development (Fig. 5D). In this analysis, while the phenotype tended to be rescued by Wdr45R, the effects were not statistically significant (Fig. 5D). Collectively, we concluded that Wdr45-deficiency impairs neuronal connectivity through defective axon elongation as well as aberrant dendrite arborization.

Role of Wdr45 in spine morphology regulation *in vivo*

As a next set of experiments, we examined the physiological role of Wdr45 in the spine density and morphogenesis *in vivo*. To this end, pSuper-H1.shLuc (control), pSuper-Wdr45#1 or #2 was co-

electroporated at E14.5 with pCAG-loxP-GFP plus pCAG-M-Cre for sparse GFP-labeling. Brains were then fixed and analyzed at P10. Consequently, the total spine density was found to be significantly decreased when Wdr45 was silenced (Fig. 6A and B, *left*). The abnormal phenotype by pSuper-Wdr45#1 was rescued by co-expression of Wdr45R (Fig. 6A and B, *left*). In addition, silencing of Wdr45 caused reduction of mature mushroom-type spine number (Fig. 6B, *middle* and *right*). The phenotype by pSuper-Wdr45#1 was rescued by Wdr45R (Fig. 6B, *middle* and *right*). These results strongly suggest that Wdr45 plays a pivotal role in the synapse formation and/or maintenance.

Long term effects of Wdr45-knockdown on neuronal morphology *in vivo*

Although individuals with BPAN develop sudden-onset progressive dystonia, parkinsonism and dementia in adolescence, pathophysiological mechanism(s) underlying these clinical manifestations is yet to be clarified. To gain some insight into this aspect, we examined long-term effects of Wdr45-knockdown on the neuronal morphology by focusing on dendritic arbor development at P30, P60 and P90. To this end, we measured the intersection number of dendritic arbor as well as the total length of apical and basal dendrites. As shown in Fig. 7A - E, dendritic arbor complexity of Wdr45-deficient neurons remained poor when compared to the control cells at the abovementioned 3 time points. Likewise, both apical and basal dendritic length of the deficient neurons were shorter than those of the control cells at the time points (Fig. 7F and G). These results strongly suggest that Wdr45-deficiency prevents neuronal network formation even in the adult stage and causes sustained impairment of the synaptic function.

We then looked into the long-term effects of Wdr45-knockdown on spine density and morphology of layer II-III cortical neurons *in vivo*. While the reduced total spine density was still observed at P30, it was comparable to the control neurons at P60 (Fig. 8A and B, *left*). In contrast, the number of mature mushroom-type spine remained to be decreased at P60 (Fig. 8A and B, *middle*).

WDR45-deficiency has been reported to downregulate autophagy in adult mice^{9,10} and LCLs from BPAN patients⁴. We asked whether silencing of Wdr45 affects the autophagy pathway during cortical development. Since LC3 (microtubule-associated light chain 3)-II is a standard marker for autophagosomes and relatively specifically associated with nascent autophagosomes, quantification of LC3-positive puncta is crucial for assessing the numbers of autophagosomes in cells. Thus, the effect of Wdr45-silencing on puncta formation of LC3 was examined in dendrites of primary cultured cortical neurons, since the main site of autophagosome formation in neurons is dendrites¹¹. Consequently, the staining pattern of LC3 in the deficient neurons was comparable to the control cells (Supplementary Fig. 3).

Discussion

Autophagy is a basic self-digestion process regulating the intracellular degradation, where cytoplasmic materials are packaged into autophagosomes and delivered to lysosomes for degradation^{2,12}. Autophagy is a key regulatory system for cell growth, differentiation, cell survival and physiological

homeostasis ^{1,2,13}, and is thought to be impaired in various neurodegenerative disorders ^{12,14}. Given that autophagy is involved in synaptic remodeling and plasticity in neurons ^{11,15}, disruption of autophagy is supposed to cause disturbed neuronal functions related to symptoms of BPAN, such as psychomotor retardation and ID in early childhood ¹⁶. Since ID has been considered to be generally related to the dysfunction of molecules involved in the synaptic function, WDR45 is assumed to play an important role in the synaptic structure and/or function during brain development. In this context, it is of note that a large-scale genomic study of individuals with undiagnosed and severe developmental disorders revealed *de novo* mutations in *WDR45*¹⁷. We thus explored the role of WDR45 during corticogenesis, using an acute gene transfer method into embryonic mouse brain. We first determined that Wdr45 is distributed in the neuronal synapses in biochemical and immunohistochemical analyses, implicating a specific role of Wdr45 at postsynapses. Then, since *WDR45* mutations responsible for BPAN are most likely to cause unstableness of WDR45 ⁴ and subsequent degradation, we carried out mouse experiments with acute gene knockdown of Wdr45 *in utero* in order to mimic the pathophysiological conditions of BPAN. Consequently, WDR45-deficiency was shown to cause defects in dendritic arborization as well as synaptogenesis of cortical excitatory neurons during brain development. Axon pathfinding was also impaired as to extension into the cortical layer structure after normally reaching contralateral hemisphere. However, not only the pathophysiological mechanism of *WDR45* gene abnormality but also molecular machinery downstream of Wdr45-mediated signaling is yet to be elucidated.

Impaired neuronal functions are also thought to cause other clinical features of BPAN including sudden-onset of progressive dementia and parkinsonism in adolescence. Although *WDR45* gene abnormalities are responsible for these clinical features in adolescence, its molecular pathophysiological mechanism also remains unknown. Consistent with the essential role of WDR45 in autophagy (X), several reports have shown that some BPAN symptoms in the adult phase, such as learning impairment and memory disturbance, are due to defective autophagy ^{10,18}.

As for the role of Wdr45 in autophagy, constitutive Wdr45-knockout (KO) mouse demonstrated apoptosis-mediated neuronal loss in several brain regions, such as prefrontal cortex and basal ganglion in aged mouse ⁹, whereas the central nervous system-specific Wdr45-KO mouse exhibited less characteristic autophagy phenotype; any significant change was not observed in the number of neuronal cells in cerebellum and hippocampus ¹⁰. Instead, these conditional KO mice displayed scattered remarkable axonal swelling in various brain regions including cerebral cortex and cerebellum, as well as reactive astrogliosis in various brain regions including hippocampus ¹⁰. From these results, Wdr45-deficiency is involved in varying degree of neuronal damage in mouse brain, although underlying molecular mechanism is enigmatic. In our preliminary analysis, while WDR45-knockdown caused long term effects on neuronal cell morphology including axon, dendrite and spine, the knockdown little affected the expression profile of an autophagy-related molecules, LC3, in primary cortical neurons. Thus, pathophysiological significance of autophagy in the abnormal morphological phenotypes observed in this study remains to be clarified. Further intensive investigation is essential to clarify the molecular aspect of functional defects of WDR45 as well as the relationship of hampered autophagy with BPAN

etiology. It is possible that other Wdr45 homologs (Wipi1 – 3) compensate for the deficiency of Wdr45 in a context-dependent manner, although further examination is required to address this issue. In addition, given that some autophagy proteins participate in autophagy-independent processes¹⁹, results in this study might suggest yet unidentified Wdr45 function which is independent of autophagy.

In conclusion, the present study links the function of Wdr45 to dendritic arborization, synaptogenesis and axon elongation of cortical excitatory neurons. We assume that WDR45-deficiency affects functions of yet unidentified proteins involved in cytoarchitecture formation/maintenance such as cytoskeletal proteins and cell signaling molecules, and is associated with pathogenicity of neurodevelopmental symptoms of BPAN. Further investigation is essential to determine whether or not the Wdr45 functions observed here are indeed dependent on the autophagy pathway.

Materials And Methods

Ethics approval and consent to participate

We followed the fundamental guidelines for proper conduct of animal experiments and related activity in academic research institution under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. All the protocols for animal handling and treatment were reviewed and approved by the animal care and use committee of Institute for Developmental Research, Aichi Developmental Disability Center (approval number M-10). All experiments were conducted in compliance with the ARRIVE guidelines. All methods, data presentation and statistical analyses were carried out in accordance with the 'TOP' guidelines. This study has not been pre-registered.

Plasmids

Mouse (m) *Wdr45* was amplified by PCR and subcloned into pCAG-Myc vector (Addgene Inc., Cambridge, MA). For RNAi experiments, following target sequences were inserted into pSuper-puro vector (OligoEngine, Seattle, WA): mWdr45#1, CAAGAAAGCTGTTTGAGTT (395 - 413); mWdr45#2, CCCTTATTCGTCTCTTTGA (644 - 662). Numbers indicate the positions from Wdr45 translational start sites. We named these vectors as pSuper-Wdr45#1 and #2. For the control RNAi experiments, pSuper-H1.shLuc designed against luciferase (CGTACGCGGAATACTTCGA) was used. To generate RNAi-resistant Wdr45, Wdr45R, silent mutations were introduced, as underlined, in the target sequence (CAAGGAAATTATTCGAGTT in mWdr45#1). The Wdr45 mutant related to BPAN pathogenicity⁶, c.700C > T/p.(Arg234*) (Wdr45-Rstop), was prepared using KOD-Plus Mutagenesis kit (Toyobo Inc., Osaka, Japan). Likewise, another mutant with a single amino acid substitution, c.439G>T/p.(Gly167Val) (Wdr45GV), was prepared. All constructs were verified by DNA sequencing.

Primary antibodies

Polyclonal rabbit anti-WDR45 antibody was kindly provided by Dr. H. Saitsu (Hamamatsu Medical University, Japan). Polyclonal rabbit anti-GFP and anti-Myc were prepared and characterized previously

²⁰. Polyclonal rabbit anti-SEPT11 was prepared as described ²¹. Monoclonal mouse anti-GFAP (Sigma-Aldrich, St. Louis, MO, USA), anti-synaptophysin (Progen Biotechnik, Heidelberg, Germany), anti-PSD95 (Thermo Scientific, Waltham, MA), monoclonal mouse anti-Myc (Cell signaling, Nagoya, Japan) and anti-LC3 (COSMO BIO, Tokyo, Japan) antibodies were also used.

Preparation of extracts from mouse tissues and western blotting

Sample preparation was performed essentially as described ^{22–24}. Briefly, tissues from ICR adult mice (Japan SLC, Shizuoka, Japan) were homogenized with the lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1% NaF, 5 mM EDTA, 1 mM Na₃VO₄ and the protease inhibitor mix). Each suspension was sonicated on ice and solubilized with the lysis buffer containing 2% SDS. Protein concentration was estimated with BCA protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. SDS-PAGE (10 % gel) and western blotting were performed as described ^{22–24}.

Preparation of postsynaptic density

Sample preparation was performed essentially as described ²³. Briefly, adult mouse brains were homogenized in 5 mM HEPES (pH 7.4) containing 1 mM MgCl₂, 0.5 mM CaCl₂, phosphatase inhibitors and the protease inhibitor mix with a Teflon homogenizer (H fraction). The resulting extract was centrifuged at low speed (1,400 x *g* for 10 min) to obtain S1 as a supernatant fraction. S2 fraction was then obtained by centrifugation of S1 at 13,800 x *g* for 10 min. The resulting pellet (P2) was resuspended in 6 mM Tris (pH 8.0) containing 0.32 M sucrose, loaded onto a discontinuous sucrose gradient, and centrifuged at 82,500 x *g* for 2 h. The synaptosome fraction was collected, extracted with 0.5 % Triton X-100 and centrifuged at 32,800 x *g* for 20 min. The resulting pellet (PSD-I; crude PSD fraction) was again extracted with 0.5% Triton X-100 and centrifuged again at 201,800 x *g* for 1 h to obtain highly purified PSD fraction as pellet (PSD-II).

Immunohistochemical analyses

Mouse brains of either sex were fixed with 4% paraformaldehyde at various time points and cut into coronal sections (6-μm thickness) at the level of dorsal hippocampus. After deparaffinization, sections were processed for immunohistochemistry as reported previously ^{21,22}. Images were captured using BZ-9000 microscope (Keyence Inc., Osaka, Japan).

Cell culture, transfection and immunofluorescence

COS7 cell transfection experiments were performed with polyethyleneimine “MAX” reagent (Polysciences Inc., Warrington, PA). Immunofluorescence analyses were carried out as described ^{22–24}. Alexa Fluor 488-, 594- or 647-labeled IgG (Life Technologies) was used as a secondary antibody. Fluorescent images were captured with a Zeiss880 confocal microscopy.

***In utero* electroporation and analysis of neuronal migration**

In utero electroporation was carried out at E14.5 and analyses were conducted at P2 essentially as described²². E14.5 ICR mice were housed individually given *ad libitum* access to food and water. Both female and male pups were used. Pups were maintained in a 12:12 light-dark cycle together with their dams before being sacrificed at indicated time points, which were primary endpoints of this study. At least 5 electroporated brains from different dams were used for each experiment. After anesthetization of pups on ice for 5 to 10 min affording physical relief, brains were fixed overnight in 4% paraformaldehyde phosphate buffer solution. Sections (100 μ m- and 150 μ m-thickness for P2 and above P10 samples, respectively) were prepared by vibratome. Distribution of GFP-positive neurons was then analyzed as described^{25,26}.

Quantitative analysis of dendritic arbor formation

To measure the length and branching point number of dendrites of post-migratory (mature) neurons, images of GFP-positive neurons in the layer II-III of cerebral cortex at P10, P30, P60 or P90 were acquired by a Zeiss880 confocal microscopy. Image J software was used for the quantitative analyses of dendritic length and Sholl test.

Quantitative analysis of spine morphology

Electroporated neurons were visualized by immunostaining of co-transfected GFP with anti-GFP. Electroporated cells were chosen randomly and images were obtained using a Zeiss880 confocal microscope. We took z-series stacks (0.2 μ m-thickness) and used Zen software to generate image projections for quantitative analyses. Morphometric measurements were automatically performed using ImagePro PLUS software. To analyze spine density and morphology, more than 200 spines from 20 or more neurons were measured for each condition. Morphological assessments were conducted blindly.

Statistical analysis

Data were analyzed using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA). Results were expressed as means $\pm$ SD. When data were obtained from only 2 groups, Student's *t*-test was used for comparison. Values are expressed as means $\pm$ SEM. For other experiments, the rate of cell scores was initially analyzed using the one-way analysis of variance (ANOVA) with Tukey's or Dunnett's *post hoc* test for multiple comparisons. The level of statistical significance was considered to be $p < 0.05$.

Declarations

Authors' contributions

M.N. participated in the design of the study and carried out *in utero* electroporation and immunohistochemical analyses. H.I. performed *in vitro* spine morphology analyses. K.N. conceived of the study, participated in its design and coordination, and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would thank Prof. Hiroto Saito (Yokohama City Univ., Yokohama, Japan) for anti-WDR45.

Additional information

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported in part by JSPS KAKENHI Grant (grant numbers; 24390271, 23390275, 26893252, 17k16315, 20k08271).

References

1. Codogno, P. & Meijer, A. J. Autophagy and signaling: Their role in cell survival and cell death. *Cell Death Differ.***12**, 1509–1518 (2005).
2. Mizushima, N. & Komatsu, M. Autophagy: Renovation of cells and tissues. *Cell***147**, 728–741 (2011).
3. Grimm, M., Backhaus, C. & Proikas-Cezanne, T. WIPI-Mediated Autophagy and Longevity. *Cells***4**, 202–217 (2015).
4. Saito, H. *et al.* De novo mutations in the autophagy gene WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood. *Nat. Genet.***45**, 445–449 (2013).
5. Chowdhury, S. *et al.* Insights into autophagosome biogenesis from structural and biochemical analyses of the ATG2A-WIPI4 complex. *Proc. Natl. Acad. Sci.***115**, E9792–E9801 (2018).
6. Hayflick, S. J. *et al.* Beta-propeller protein-associated neurodegeneration: A new X-linked dominant disorder with brain iron accumulation. *Brain***136**, 1708–1717 (2013).
7. Tsuyuki, S. *et al.* Detection of WIPI1 mRNA as an indicator of autophagosome formation. *Autophagy***10**, 497–513 (2014).
8. Vissers, L. E. L. M. *et al.* Genetic studies in intellectual disability and related disorders. *Nat. Rev. Genet.***17**, 9–18 (2015).

9. Wan, H. *et al.* WDR45 contributes to neurodegeneration through regulation of ER homeostasis and neuronal death. *Autophagy***16**, 531–547 (2020).
10. Zhao, Y. G. *et al.* The autophagy gene Wdr45/Wipi4 regulates learning and memory function and axonal homeostasis. *Autophagy***11**, 881–890 (2015).
11. Nixon, R. A. The role of autophagy in neurodegenerative disease. *Nat. Med.***19**, 983–997 (2013).
12. Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. *Nature***451**, 1069–1075 (2008).
13. Harris, H. & Rubinsztein, D. C. Control of autophagy as a therapy for neurodegenerative disease. *Nat. Rev. Neurol.***8**, 108–117 (2012).
14. Behrends, C., Sowa, M. E., Gygi, S. P. & Harper, J. W. Network organization of the human autophagy system. *Nature***466**, 68–76 (2010).
15. Shehata, M., Matsumura, H., Okubo-Suzuki, R., Ohkawa, N. & Inokuchi, K. Neuronal stimulation induces autophagy in hippocampal neurons that is involved in AMPA receptor degradation after chemical long-term depression. *J. Neurosci.***32**, 10413–10422 (2012).
16. Ji, C. *et al.* Role of Wdr45b in maintaining neural autophagy and cognitive function. *Autophagy***16**, 615–625 (2020).
17. McRae, J. F. *et al.* Prevalence and architecture of de novo mutations in developmental disorders. *Nature***542**, 433–438 (2017).
18. Paudel, R. *et al.* Neuropathology of Beta-propeller protein associated neurodegeneration (BPAN): a new tauopathy. *Acta Neuropathol. Commun.***3**, 39 (2015).
19. Subramani, S. & Malhotra, V. Non-autophagic roles of autophagy-related proteins. *EMBO Rep.***14**, 143–151 (2013).
20. Mizutani, Y. *et al.* Possible role of a septin, SEPT1, in spreading in squamous cell carcinoma DJM-1 cells. *Biol. Chem.***394**, 281–290 (2013).
21. Hanai, N. *et al.* Biochemical and cell biological characterization of a mammalian septin, Sept11. *FEBS Lett.***568**, 83–88 (2004).
22. Noda, M. *et al.* Role of Per3, a circadian clock gene, in embryonic development of mouse cerebral cortex. *Sci. Rep.***9**, 5874 (2019).
23. Ito, H., Morishita, R., Noda, M., Iwamoto, I. & Nagata, K. ichi. Biochemical and morphological characterization of SEPT1 in mouse brain. *Med. Mol. Morphol.***53**, 221–228 (2020).
24. Ibaraki, K. *et al.* Biochemical and morphological characterization of a guanine nucleotide exchange factor ARHGEF9 in mouse tissues. *Acta Histochem. Cytochem.***51**, 119–128 (2018).
25. Nishimura, Y. V., Shinoda, T., Inaguma, Y., Ito, H. & Nagata, K. I. Application of in utero electroporation and live imaging in the analyses of neuronal migration during mouse brain development. *Medical Molecular Morphology***45**, 1–6 (2012).
26. Tabata, H. & Nakajima, K. Efficient in utero gene transfer system to the developing mouse brain using electroporation: Visualization of neuronal migration in the developing cortex. *Neuroscience***103**, 865–

Figures

Fig.1 Noda *et al.*

Figure 1

Expression profiles of Wdr45 in mouse tissues (A) Whole lysates (30 µg protein per lane) of various adult mouse tissues were subjected to western blotting (10 % gel) with anti-WDR45. Molecular weight markers are shown at the left. (B) Whole brain lysates (30 µg protein per lane) at indicated developmental stages were subjected to western blotting with anti-WDR45. Anti-GFAP and anti-SEPT11 were used for a differentiation marker and a loading control, respectively. (C) Subcellular fractions (30 µg protein per lane) prepared from adult forebrains by differential and density-gradient centrifugation were subjected to western blotting with anti-WDR45. Anti-synaptophysin (SYP) and anti-PSD95 were used for presynaptic and postsynaptic markers, respectively. Hom, homogenate; S1, soluble fraction; S2, crude cytosol fraction; P1, nuclear fraction; P2, crude membrane fraction; Syp, synaptosomal fraction; PSDI, PSD fraction-I; PSDII, PSD fraction-II

Fig.2 Noda *et al.*

Figure 2

Immunohistochemical analysis of Wdr45 in mouse cerebral cortex. (A) Coronal sections of the somatosensory area at E15 (a), E17 (b), P0 (c), P7 (d) and P30 (e) were stained with anti-WDR45. VZ, ventricular zone; SVZ, subventricular zone; IMZ, intermediate zone; SP, subplate; CP, cortical plate; MZ, marginal zone; WM, white matter. (B) Cells at the indicated areas in (A, a-c) were magnified. (C) Cells at the indicated areas in (A, d and e) were magnified. (D) An area of Layer II/III in the cortex at P30 was

shown. Counter staining was performed using Mayer's hematoxylin. Scale bars, 50 μ m (A) and 10 μ m (B-D).

Fig.3 Noda *et al.*

Figure 3

Characterization of vectors for expression and RNAi. (A) Expression of wild type Wdr45 and 2 mutants, c.700C > T/p.(Arg234*) (Wdr45-Rstop) and c.439G>T/p.(Gly167Val) (GVmut), in dissociated mouse cortical neurons. Neurons isolated at E14 were transfected with pCAG vector (control), pCAG-Myc-Wdr45

(WT), -WDR45-GVmut or -WDR45-Rstop, and cultured in vitro for 13 days. Cells were then subjected to SDS-PAGE followed by western blotting with anti-Myc. Anti-SEPT11 was used for a loading control. (B) Knockdown of exogenous Wdr45 in COS7 cells. pCAG-Myc-Wdr45 was co-transfected with pSuper-H1.shLuc (Control), pSuper-Wdr45#1 (KD#1) or #2 (KD#2). After 48 h, cells were harvested and subjected to western blotting with anti-Wdr45 or anti-Myc. Anti-SEPT11 was used for loading control. (C) Knockdown of endogenous Wdr45. pCAG-EGFP was co-transfected with pSuper-H1.shLuc (Control), pSuper-Wdr45#1 or #2 into dissociated cortical neurons as in (A), and cultured for 72 h. After fixation, cells were immunostained with anti-GFP (green) and anti-WDR45 (red). Scale bars, 50 μ m. (D) Characterization of RNAi-resistant Wdr45. pCAG-Myc-Wdr45 or pCAG-Myc-Wdr45R was co-transfected into COS7 cells with pSuper-H1.shLuc (Control), pSuper-Wdr45#1 or #2. After 48h, cells were subjected to western blotting as in (B).

Fig.4 Noda *et al.*

Figure 4

Role of Wdr45 in the callosal axon growth in vivo. (A) pCAG-Turbo-RFP was co-electroporated with pSuper-H1.shLuc (control), pSuper-Wdr45#1 or #2 into the VZ progenitor cells at E14.5 and fixed at P2. Coronal sections were then prepared and analyzed. Representative images of callosal axons expressing RFP were shown. Note that Wdr45-deficiency less affected the axon growth at this timing. (B and C) Effects of Wdr45-knockdown on axon extension into the contralateral cortex at P30 (B) or P60 (C).

Representative images of the terminal arbors of callosal axons expressing RFP with pSuper-H1.shLuc or pSuper-Wdr45#1 were shown. Quantitative densitometric analyses of RFP fluorescence intensity were performed for axons extending into the contralateral cortical layer structure of control (black line) and Wdr45-deficient (red line) neurons. Distance from the VZ surface along double sided arrows represents the vertical (y) axis. Shadows represent SD [control, n = 3 and KD#1, n = 6 for (B); control, n = 3 and KD#1, n = 5 for (C)]. Scale bars, 50 mm (A) and 500 μ m (B).

Fig.5 Noda *et al.*

Figure 5

Effects of Wdr45-knockdown on dendrite growth in vivo. (A) For sparse expression, pCAG-loxP-GFP (0.5 μ g) was co-electroporated with pCAG-M-Cre (1 ng) together with pSuper-H1.shLuc (control), pSuper-Wdr45#1, #2 or pSuper-Wdr45#1 plus pCAG-Myc-Wdr45R (1.5 μ g each of pSuper vector and 0.5 μ g of pCAG-Myc-Wdr45R) at E14.5. Coronal sections were prepared, stained for GFP and analyzed at P10. Representative average z-stack projection images of layer II/III cortical neurons were shown. Scale bars, 50 μ m. (B) Branch points of dendrites in (A) were quantified by Sholl test. One section from each brain was analyzed for control (n=3), KD#1 (n=7), KD#2 (n=6), and KD#1 + Wdr45R (n=5). Error bars indicate SD of the results from 10 to 15 neurons; ****P < 0.0001, by one-way ANOVA with Dunnett's post hoc test (vs. control). (C and D) Total length was calculated for apical (C) and basal dendrites (D). #P < 0.05, ***P < 0.001 and ****P < 0.0001 (*, vs control; #, vs KD#1) by one-way ANOVA with Tukey's post hoc test.

Fig.6 Noda *et al.*

Figure 6

Effects of Wdr45-knockdown on the dendritic spine density and morphology in vivo. (A) pCAG-loxP-GFP was electroporated with pCAG-M-Cre together with pSuper-H1.shLuc (Control), pSuper-Wdr45#1 (KD#1), #2 (KD#2) or KD#1 plus pCAG-Myc-Wdr45R at E14.5. Coronal sections were prepared at P10 and stained for GFP. Representative images of dendritic spines on the 1st-order apical dendrites were shown. Scale bars, 5 μm. (B) Quantitative analyses of spine density and morphology (mature mushroom-shaped;

immature filopodia-like or stubby spines) in layer II/III cortical excitatory neurons. One section from each brain was analyzed for control (n=3), KD#1 (n=4), KD#2 (n=4) and KD#1 + Wdr45R (n=5). Error bars indicate SD of the results from 20 to 30 neurons; *P < 0.05, ****P < 0.0001 by one-way ANOVA with Dunnett's post hoc test (vs. control).

Fig.7 Noda *et al.*

Figure 7

Long-term effects of Wdr45-knockdown on dendrite growth in vivo. (A, B) For sparse expression, pCAG-loxP-GFP was co-electroporated with pCAG-M-Cre together with pSuper-H1.shLuc (control) (A) or pSuper-Wdr45#1 (KD#1) (B) at E14.5. After fixation at P30, P60 or P90, coronal sections were prepared and stained for GFP. Representative average z-stack projection images of layer II/III cortical neurons were shown. Scale bars, 50 μ m. (C - E) Branch points of dendrites were analyzed by Sholl test. One section from each brain was analyzed for P30 (control, n=10; KD#1, n=11) (C), P60 (control, n=6; KD#1, n=8) (D) and P90 (control, n=3; KD#1, n=3). Error bars indicate SD of the results from 10 to 15 neurons; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 by t-test (vs. control). (F and G) Total dendritic length was calculated for apical (F) as well as basal dendrites (G). Error bars indicate SD of the results from 10 to 15 neurons; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 by t-test (vs. control).

Fig.8 Noda *et al.*

Figure 8

Long-term effects of Wdr45-knockdown on dendritic spine density and morphology in vivo. (A) pCAG-loxP-GFP was co-electroporated with pCAG-M-Cre together with pSuper-H1.shLuc (Control) or pSuper-Wdr45#1 (KD#1) at E14.5. After fixation at P30 or P60, coronal sections were stained for GFP. Representative images of dendritic spines on the 1st-order apical dendrites were shown. Scale bars, 5 μ m. (B) Quantitative analyses of spine density and morphology were performed as in Fig. 6A. One section

from each brain was analyzed for P30 (control, n=5; KD#1, n=6) and P60 (control, n=4; KD#1, n=5). Error bars indicate SD of the results from 20 to 30 neurons; ****P < 0.0001, ###P < 0.001 by one-way ANOVA with Tukey's post hoc test (*, vs control; #, vs KD#1).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryDataset2.pdf](#)