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概要書

Molecular mechanisms regulating brain development
脳構築機構を制御する分子メカニズムの解明

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The spatiotemporal differentiation of neural stem/progenitor cells (NSPCs) into immature neurons and neuronal migration are necessary for the proper development of the central nervous system (CNS). The cerebral cortex of embryonic mammals contains two distinct types of NSPCs: apical progenitor cells, located in the ventricular zone (VZ), and basal progenitor cells, located in the subventricular zone (SVZ) (Englund et al., 2005). In the neocortex, newborn neurons generated from NSPCs migrate radially toward the cortical plate, accompanied by sequential changes in cell shape. Neurite outgrowth and ensuing polarity formation in immature neurons are also required for cortical layer stratification, and defects in neuronal migration not only cause brain malformation but also various psychiatric disorders, including epilepsy and mental retardation (Hansen et al., 2017; Represa, 2019).

Purines are found in all living species and include the nucleobases adenine and guanine (Traut, 1994). In mammalian cells, purine content is regulated by a coordinated balance between the de novo and salvage biosynthetic pathways. Although the cellular purine pool is usually supplied by the recycling of degraded bases via the salvage pathway, the de novo pathway is upregulated under cellular conditions demanding higher levels of purines and their derivative nucleotides, such as tumor growth and cell proliferation (Yamaoka et al., 1997). De novo purine synthesis comprises a series of 10 enzymatic reactions and is mediated by six evolutionarily conserved enzymes (PPAT, GART, FGAMS, PAICS, ADSL, and ATIC) (Baresova et al., 2018). The enzymes that catalyze de novo purine synthesis are assembled near mitochondria and microtubule as a huge multienzyme complex called ‘‘purinosome’’ (An et al., 2008, 2010; French et al., 2016). Purinosome is a dynamic and functional giant protein complex that emerges during high levels of cellular purine demand in mammalian cultured cells (An et al., 2008). The dynamic assembly and disassembly of purinosomes *in vivo* might be crucial for the proper development of the human brain. Mutations in ADSL and ATIC genes cause severe developmental brain defects, such as mental retardation, autistic features, epilepsy, microcephaly, and congenital blindness (Jurecka et al., 2015; Marie et al., 2004). PAICS is associated with prostate and breast cancer metastasis and proliferation (Barrfeld et al., 2015; Chakravarthi et al., 2018; Meng et al., 2018). PAICS mutations in humans causes the severe phenotype with multiple malformations, including a small body, short neck, and craniofacial dysmorphism, resulting in early neonatal death (Pelet et al., 2019). To date, however, there is no direct evidence of the localization or physiological function of purinosomes during brain development.

To isolate genes that are important for NSPCs, we used the suppression subtractive hybridization (SHH) technique, by which we identified several clones encoding for novel proteins or proteins of unknown function in NSPCs. Here, we focused on the characterization of NACHT and WD repeat domain-containing protein 1 (Nwd1). A BLAST homology search revealed Nwd1 is highly conserved across species, including, mice, rats, cattle, monkeys, and humans. Motif search analysis showed that Nwd1 protein contained a NACHT domain in the central region, followed by a cluster of 12 WD40 repeats at the C-terminus. Based on recent sequence-based structure predictions, many genes containing a P-loop NTPase signature NACHT domain have been classified as the clade of STANDs (Leipe et al., 2004). By comparing the domain architecture, we found that Nwd1 was most similar to apoptotic peptidase activating factor 1 (Apaf1), due to the presence of the clustered WD40 repeats, in the C-terminal region, as well as of the centrally located NACHT domain. However, the N-terminal region of Nwd1 lacked the CARD found in Apaf1, as well as the well-characterized effector domains commonly found in numerous other STAND proteins. We hypothesized that this could infer the existence of an unidentified and unique effector domain in Nwd1. Indeed, using the InterPro database search (Finn et al., 2017), we found a short stretch of sequence, in the N-terminal region of Nwd1, which exhibited similarity to domain of unknown function DUF4062.

To examine the expression of Nwd1 in NSPCs *in vivo*, we performed in situ hybridization analysis using tissue from developing and postnatal brains. A Strong *Nwd1* expression was observed in the VZ and SVZ surrounding the ventricles where NSPCs were localized. To assess the tissue localization of Nwd1 protein, we raised a polyclonal antibody against the mouse protein. Nwd1 was intensely expressed in the cytoplasm and

cellular processes of actively proliferating NSPCs. In addition, we found that Nwd1 localized with cytochrome c, a marker of mitochondria, in NSPCs. To assess the spatiotemporal distribution of Nwd1, we performed immunohistochemistry in the developing and adult mice brain. Strong Nwd1 expression was observed in NSPCs and immature neurons in the developing cerebral cortex. In contrast, Nwd1 signals were observed in the matured neuron in the adult mice brain. These results indicate the abundance of Nwd1 mRNA and protein in NSPCs populations in the developing cerebral cortex.

We next investigated the role of Nwd1 in the developing cerebral cortex, we overexpressed and knockdown the Nwd1 gene *in vivo* using *in utero* electroporation. Nwd1 overexpression maintained NSPCs populations and delays neuronal migration. Nwd1 knockdown also suppressed neuronal migration in VZ/SVZ and IZ due to defects on polarity formation in the migrating neurons. Doble immunostaining revealed Nwd1 knockdown cells were exited from apical progenitor cells prematurely and became basal progenitor cells or immature neurons. Accordingly, Nwd1 knockdown decreased NSPCs proliferation. These results were also observed in *in vitro* experiments using primary cultured NSPCs and cortical neurons. We examined postnatal brains, when neocortex stratification is almost complete, after the embryonic knockdown of Nwd1. We observed Nwd1 knockdown pups frequently developed periventricular heterotopia (PH) manifested by ectopic nodular masses in the lining of the ventricular wall. Indeed, double immunostaining revealed these cells in PH were glutamatergic neuron. Cellular architecture of this malformation was similar to the human PH composed of hyperexcitable neurons, which is a developmental cortical dysgenesis frequently characterized by focal drug-resistant epilepsy (Battaglia et al., 2006).

We attempted to understand the molecular mechanism by which Nwd1 regulates cortical development. Based on its structural similarity to other STAND-family proteins (Leipe et al., 2004), we hypothesized that the N-terminal region of Nwd1 serves as an effector domain by which the protein binds signaling molecule(s) to trigger self-oligomerization mediated by the NACHT domain and WD40 repeats. Therefore, we used a yeast two-hybrid screen to identify proteins interacting with N-terminal region of Nwd1. The screening of a mouse brain library led to the isolation of 14 putative Nwd1-binding partners, including three independent Paics cDNA clones. We hypothesized that Nwd1 is involved in the formation of the purinosome. We examined the colocalization of Nwd1 with Paics or Fgams, both of which are used as purinosome markers. HeLa cells that expressed Flag-Nwd1 and Paics-EGFP or Fgams-EGFP transiently were cultured in purine-depleted media, to induce e formation of cellular purinosomes. We observed many purinosomes became evident as the cytoplasmic clustering of Fgams-EGFP or Paics-EGFP along with Nwd1.

We next investigated whether NSPCs can form purinosomes and whether Nwd1 localizes in these structures in NSPCs. The expression of Fgams-EGFP distinctly emerged as a granular structure. Double immunostaining indicated that a significant proportion of the endogenous Paics or Nwd1 protein colocalizes in these clusters. To examine the role of Nwd1 in purinosome assembly, Nwd1 shRNA constructs were electroporated into NSPCs expressing Fgams-EGFP. Nwd1 knockdown reduced the number of cells containing Fgams- $EGFP^+$ $Pacis^+$ purinosomes considerably. Because a protein complex lacking Paics no longer functions as a purinosome, we concluded that Nwd1 is required for the assembly of the functional purinosome in NSPCs.

To clarify the involvement of the purinosome in brain development, we examined the loss-of-function or gain-of-function phenotypes of Paics and Fgams *in utero*. We revealed that Paics loss of function suppressed neuronal migration. Double immunostaining indicated Paics knockdown induced mitotic exit and premature differentiation of NSPCs. In contrast, Fgams overexpression significantly suppressed neuronal migration from the VZ, leading to the accumulation of Fgams-overexpressing cells as $Nestin^+$ NSPCs in the VZ/SVZ.

Taken together, these data provide strong evidence that both Paics and Fgams are essential for neurogenesis and corticogenesis and that the dysregulation of these genes hinders neuronal migration. Such abnormal properties of neurons and NSPCs caused by the manipulation of Paics and Fgams seemed to be a phenocopy of Nwd1 overexpression/knockdown. The de novo biosynthesis of purines, especially the tightly regulated levels of purinosome components, is indispensable for the orchestrated migration and differentiation of neurons that occur during brain development. In humans, PH is associated with intractable epilepsy and intellectual disability (Cossu et al., 2018). Thus, the disturbance of the purine de novo synthesis pathway may be associated, at least in part, with the mechanism underlying the pathogenesis of PH.

In this study, we revealed the molecular mechanism of brain development by the novel gene Nwd1 involved in a variety of human diseases including psychiatric disorders, epilepsy, and cancer. Our research is expected to contribute to the elucidation of the pathogenesis of these diseases and the development of therapeutic drugs targeting Nwd1.