Cnpy3 2xHA mice reveal neuronal expression of Cnpy3 in the brain

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論文題目

Cnpy3^{2xHA} mice reveal neuronal expression of Cnpy3 in the brain

（*Cnpy3*^{2xHA} マウスを用い Cnpy3 が神経細胞に発現していることを明らかにした）

論文の内容の要旨

[Introduction]

Cnpy3 (Canopy FGF Signaling Regulator 3) encodes a protein known as Canopy3 or PRAT4A is known to be a chaperone for the regulation of folding and trafficking of Toll-like receptor 4 (TLR4) in the immune cells and localizes in the endoplasmic reticulum (ER). It is also known to form a complex with heat shock protein gp96 and serve as a chaperone of multiple TLRs. In addition, the *Cnpy3* knock-out mouse has previously been reported to show the decreased surface expression of multiple TLRs on immune cells, and resistance to lipopolysaccharide administration, which induces seizures. Recently, we identified biallelic *CNPY3* mutations in patients with epileptic encephalopathy. In addition, *Cnpy3* knock-out mice showed abnormal electroencephalography, suggesting that the loss of Cnpy3 function causes neurological disorders such as epilepsy. However, the basic property of Cnpy3 in the brain remains unclear. To address these issues, we generated *Cnpy3*^{2xHA} knock-in (KI) mice and investigated the expression and function of Cnpy3 in the brain.

[Materials and Methods]

To investigate the expression of Cnpy3 in the mouse brain, we generated C-terminal 2xHA-tag KI Cnpy3 mice (*Cnpy3*^{2xHA}) by i-GONAD (improved-genome editing via oviductal nucleic acids delivery) in vivo genome editing system. For expression analysis in vitro, we constructed four different expression vectors as Cnpy3, Cnpy3 E191V, Cnpy3-2xHA, and Cnpy3 E191V-2xHA and then transfected them into the HEK293T cells using polyethyleneimine. For protein stability analysis, we performed a cycloheximide (CHX, 50mg/ml) chase assay followed by immunoblotting to analyze the time course of Cnpy3 and Cnpy3-2xHA degradation. We used both the mouse brain and cultured cell lysates for Cnpy3 protein expression analysis by immunoblotting assay. These samples were lysed with RIPA lysis buffer supplemented with the protease inhibitor and were separated by SDS–PAGE and transferred onto the PVDF membrane. Subcellular fractions were prepared from WT and *Cnpy3*$^{2xHA/2xHA}$ KI mice at 60 days of age by differential centrifugation. All centrifugations were carried out at 4℃ and immunoblotting was used to determine the subcellular distribution of the Cnpy3 protein level. To investigate which cell types express Cnpy3 in the mouse brain, we performed

immunohistochemistry using anti-HA antibodies. The brain sections were imaged using a confocal microscope. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Hamamatsu University School of Medicine (2020093, 3-25).

[Results]

HA-tagged Cnpy3 was confirmed by immunoblot analysis using HA and Cnpy3 antibodies, although HA tagging caused the decreased Cnpy3 protein level in the brain. We further investigated if this HA tagging affected the mRNA transcription of Cnpy3 using real-time quantitative PCR and found that *Cnpy3*$^{2xHA/2xHA}$ mice had a slightly increased Cnpy3 mRNA expression level in comparison to WT mice. To verify the possibility of the Cnpy3 decrease, we performed a transfection and cycloheximide chase assay using cell culture experiments and found that Cnpy3-2xHA was degraded drastically compared to Cnpy3. To investigate the expression pattern of Cnpy3-2xHA in the brain by immunohistochemistry, we employed several HA antibodies. Then, by comparing the differences in the staining pattern and the signal/background contrast between WT and *Cnpy3*$^{2xHA/2xHA}$ mouse brain sections, we confirmed the expression of Cnpy3-2xHA in the neuron. In addition, Cnpy3 was identified in all fractions obtained by differential centrifugation, but the signal was particularly strong in the heavy membrane fraction (P3HDM) and synaptosomal membrane fraction (LP1) and also showed age-dependent expression changes in the brain. These findings allowed us to demonstrate that HA tagging did not alter the localization and property of Cnpy3.

[Discussion]

Many different epitope tags are used in protein research to study protein function, protein localization, and proteome analysis. In this study, we generated a mutant mouse in which 2xHA was tagged to the C-terminus of Cnpy3. Then, we could demonstrate Cnpy3 expression in the neuron using immunohistochemistry for the first time. Furthermore, Cnpy3 expression was found to increase from the embryonic to weaning period, suggesting that it may orchestrate as one director of brain development that coordinates the expression of various TLRs involved in neurogenesis and neuronal migration. In addition, synaptosomal localization of Cnpy3 also suggests that it may regulate TLRs or other proteins involved in synaptogenesis and neuroplasticity. Although further research is necessary, this study would be a pioneering study of Cnpy3 in brain function and development.

[Conclusion]

In this study, we generated a transgenic mouse in which Cnpy3 was attached with two HA epitope tags and identified the following points. HA tagging to the C-terminal of Cnpy3 accelerated the degradation of Cnpy3 in vivo and in vitro. Cnpy3 was found to

be expressed in the neuron. Cnpy3 is expressed from the early stages of cortical development and the expression level of Cnpy3 increases during early postnatal development. Cnpy3 was enriched in the endoplasmic reticulum and synaptosome in the brain.