論文の内容の要旨

論文題目 Generation of KIF1Bbeta knockin mouse and analysis of KIF1Bbeta dynamics and function
(KIF1Bbetaノックインマウスの作成及びKIF1Bbetaの動態と機能の解析)

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Neurons are highly polarized cells consisting of soma, long axons, and dendrites. The intracellular transport of protein complexes and membrane organelles are required for neuronal functions, neurite elongation and neuronal polarization. The kinesin superfamily proteins (KIFs) play fundamental roles in intracellular transport. Kinesin Superfamily proteins (KIFs) consist of a large repertoire of microtubule binding motor proteins. 45 *Kif* genes have been identified in mouse genome, 38 of which are expressed in the brain. A KIF protein generally contains a conserved globular motor domain, a filamentous "stalk" region and a globular "tail" region. The motor domain consists of a microtubule binding domain and ATPase domain, and has a high amino acid sequence homologies of ~30-60% among KIFs. The stalk region and tail region are highly variable. KIFs bind to cargoes through their variable tail region. KIFs can be broadly grouped into 3 types according to the position of motor domain, N-kinesins carrying a motor domain in the NH_2-terminal domain, M-kinesins in the middle, and C-kinesins in the tail. KIFs are classified into 14 families according to the results of phylogenic analyses.

Kinesin-3 motors, KIF1Bα and KIF1Bβ are generated by alternative splicing of the cargo-binding domain of the *Kif1b* gene. KIF1Bα works as a motor for anterograde transport of mitochondria in axons. KIF1Bβ, which is structurally similar to KIF1A, transports synaptic vesicle precursors such as synaptophysin, synaptotagmin, and Rab3A. Previous study from our lab showed that DENN/MADD, a multifunctional domain protein expressed in neurons, has a crucial role in regulating KIF1Bβ- and KIF1A-mediated axonal transport of Rab3A-carrying synaptic precursor vesicles. Fatal neuronal defects and a significantly reduced number of synaptic vesicles were observed in *Kif1b*$^{-/-}$ mice. However, this can be rescued by KIF1Bβ but not by KIF1Bα. This suggests that KIF1Bβ is the predominant isoform of KIF1B in regulation of neural development, so I focused on research into beta-isoform.

In order to establish a new mouse model for fluorescently tagging KIF1Bβ in living neurons, I generated *Kif1bβ-EGFP* knockin mouse using gene targeting technology. Because *Kif1b* has 2 major splicing isoforms, α and β, which share an identical N-terminal region including motor domains and diverge in C-terminals. *Kif1bα* has one large specific exon, while the first splicing exon of *Kif1bβ* starts a little downstream of the α-exon. Accordingly, I decided to knockin the *EGFP* in the C-terminal of *β*-isoform.

The targeting vector was constructed so that the stop codon of *Kif1bβ* was replaced by an EGFP sequence. The long homologous arm was recovered from BAC clone using Red-ET-based homologous recombination technology in *E. coli*, which greatly improved the speed and accuracy of the DNA manipulation. Previously, promoter trapping or poly-A trapping for the positive selection cassette was applied in our lab to eliminate the non-homologous recombinant clones. However, because of the high homologous recombination efficiency in RENKA ES cell line, gene trapping could be omitted to reduce an adverse effect by reduction of the expression.

In this study I made use of C57BL/6-derived RENKA ES cell line previously established by Sakimura Lab for gene targeting for generating a kinesin-knockin model. Conventionally, ES cells derived from 129/Sv strain, such as J1 cell line, have been utilized in our lab, but it required screening and several hundred ES clones and more than 10 times of backcrossing to obtain the C57BL/6-congenic strain especially for the precise neuroscience studies. Utilizing new RENKA cell line has revolutionarily omitted these time-consuming ES screening and backcrossing steps, but its handling has much more difficulties for the specialists' sake. However, after improving the cell culture condition such as medium components, like growth factor, serum, and also cell maintenance skill, I finally succeeded in germline transmission of the recombinant allele after more than 10 trials.

On this successful electroporation, 96 colonies were picked up, 4 of which were screened out positive for homologous recombination showing the expected pattern of genomic southern blotting, so that the homologous recombination efficiency was 4.2%. Because this efficiency was significantly higher than the previous case in J1 cells, the number of picked up colonies could be greatly reduced to save the effort of researchers. Finally, one of the homologous recombinant clones made successful germline transmission, and the positive selection cassette was finally removed by crossing the mouse with *CAG-Cre* transgenic mice.

The expression of KIF1Bβ-EGFP protein was verified by immunoblotting. In the heterozygous mouse, the upper KIF1Bβ(+)-band was also positive for GFP epitopes, and was supposed to be corresponding to the KIF1Bβ-EGFP protein. Because the strengths of both KIF1Bβ(+)-bands were approximately the same, the expression level of this tagged protein may be almost consistent with that of the intrinsic protein.

Intercrossing between $Kif1b\beta^{GFP/+}$ mice delivered 43 $Kif1b\beta^{+/+}$, 69 $Kif1b\beta^{GFP/+}$, and 35 $Kif1b\beta^{GFP/GFP}$ mice, which followed the Mendelian ratio. $Kif1b\beta^{GFP/GFP}$ mice generally survived over 2 years and were fertile, which circumvented the newborn lethality of $Kif1b^{-/-}$ mice.

Acute slices of 4-week-old $Kif1b\beta^{+/+}$and $Kif1b\beta^{GFP/GFP}$ mouse brains were subjected to confocal laser scanning microscopy. Fluorescence in the hippocampal regions was compared at the excitation of 488-nm laser. $Kif1b\beta^{GFP/GFP}$ brain showed specific fluorescence signal compared with wild type control. This EGFP fluorescence signal was further confirmed by taking emission spectra, which gave a 509 nm

emission peak. This was consistent with the emission peak of EGFP, suggesting that the green fluorescence was truly derived from the knocked in EGFP protein.

By using a spinning-disc confocal unit, I took time lapse movies of dissociated culture of the knockin homozygous hippocampal neurons. In the neuronal axons, many tubulovesicular signals were mainly moving toward the axonal tips, similar to those previously observed in transfected neurons. According to its kymograph, many vesicles were observed to bidirectionally moving within axons. Quantification of the speed of anterograde vesicles revealed that the majority of anterograde vesicles were moving at 0.8–2.2 μm/s, which was consistent with the previous descriptions.

The advantage of this knockin mouse for such imaging studies will be that the recombinant KIF1Bβ is expressed under the proper control of its original promoter, which made the expression level very close to the physiological level, so that adverse effects due to protein overexpression could be eliminated. This new model mouse which I have established will be a good source for future investigations on the function and relevance of this molecular motor in physiological and pathological conditions, from the cellular to individual levels.