論文の内容の要旨

論文題目　**Molecular Genetic Study of Kinesin Superfamily Protein, KIF26A**

（モーター分子KIF26Aの分子遺伝学的研究）

氏名　王　力（Wang Li)

KIF26A is an atypical member of the kinesin superfamily proteins (KIFs). It lacks the motor ATPase activity and functions as a signaling regulator rather than a molecular motor. Here I show that mice with a homozygous deletion of *Kif26a* were suffered from hyperalgesia, showing increased sensitivity to noxious heat and mechanical stimuli. Morphologically, peripheral innervation and branching of DRG neuronal axons were enhanced. In the cultured dorsal root ganglion (DRG) neurons of *Kif26a*$^{-/-}$ mice, capsaicin-triggered $[Ca^{2+}]_i$ elevation was increased and abnormally persisted after capsaicin was washed out. Consistently, expression level and phosphorylation level of TRPV1 channel that sensitizes this capsaicin receptor, and tyrosine phosphorylation level of PMCA calcium pump that suppresses the calcium efflux, were both significantly elevated. To the upstream to these phosphorylations and abnormal development, MAPK and PI3K responses to capsaicin or NGF stimulation were significantly exaggerated in *Kif26a*$^{-/-}$ DRG neurons. KIF26A was associated with Grb2 and inhibited its interaction with SH3 in DRG cells to negatively regulate the MAPK and PI3K signal transductions. Therefore, deficiency of KIF26A resulted in the hypersensitivity of nociceptor neurons. These results propose a new role of KIF26A in the function and development of sensory neurons.

In this study I have reexamined the behavior of *Kif26a*$^{-/-}$ mice and first describe the hyperalgesia of these animals. I show that deletion of the *Kif26a* gene led to hyperalgesia, which is abnormal pain activity to noxious heat and mechanical stimuli. In situ hybridization revealed high-level expression of KIF26A in DRG neurons. Through physiological analyses of primary cultured DRG neurons, I propose a new role of KIF26A in eliminating the capsaicin-induced intracellular calcium elevation, possibly due to elevated PI3K and MAPK signaling. Furthermore, KIF26A deficiency led to peripheral innervation and branching of sensory nerves. These results suggested a new role of KIF26A in modulating the intracellular signals essential for development and function of the PANs.

According to behavioral analyses of these mice, sensory responses were significantly exaggerated in *Kif26a*$^{-/-}$ mice (KO). These results suggested that *Kif26a*$^{-/-}$mice are hypersensitive to the noxious stimuli. In order to elucidate the responsible tissue of this hyperalgesia, I examined the expression of *Kif26a* in DRG. In situ hybridization using a specific probe revealed significant expression of *Kif26a* mRNA in DRG of WT mouse

pups of postnatal 1 day old, compared with that of KO, suggesting that dysfunction of DRG neurons could cause the hyperalgesia of KIF26A-deficient mice.

Because the previous report described abnormalities in the enteric nervous system development, I investigated possible changes in development of DRG neurons. First, cryosections of *Kif26a*$^{-/-}$ and *Kif26a*$^{+/+}$ ganglia were compared using a pan-neuronal marker Islet-1. As a result, no apparent morphological changes were found at the age of postnatal 1 day. Furthermore, the total number of Islet-1^{+} neurons in T10 DRG was not significantly changed. These data suggested that KIF26A is dispensable for the regulation of the number of mouse DRG neurons at this stage.

Previous studies have shown that DRG neuron size was increased after exposure to exogenously applied NGF. Transgenic mice overexpressing NGF in skin also showed significant hypertrophy of TrkA positive neurons in DRG. To quantify the changes in neuronal size, the soma area of TrkA positive as well as the CGRP positive neurons were measured in DRG using sections from both KIF26A mutant and WT mice. The results of size-frequency analysis indicated that the size of the TrkA^{+} and CGRP^{+} neurons was increased in *Kif26a*$^{-/-}$ DRG. This result also suggested that KIF26A suppress the NGF-TrkA signaling in the DRG sensory neurons. To further study the morphological changes of *Kif26a*$^{-/-}$ DRG, I observed the innervation of free nerve ending in the footpad skin of *Kif26a*$^{-/-}$ and WT mice. The number of fibers innervating to the epidermis of *Kif26a*$^{-/-}$ footpad skin at postnatal 7 days was significantly increased compare to that of *Kif26a*$^{+/+}$skin. I next investigated whether KIF26A deficiency could affect axonal extension or branching in vivo. A whole-mount immunofluorescent staining assay using antibody against neurofilament-M was performed. I found the brancing of the NF-M positive fibers was increased in *Kif26a*$^{-/-}$ paws relative to controls at embryonic-14.5-day. The number of NF-M positive branches per nerve trunk and the number of orders of branching were both increased in KIF26A mutants compared to WT. However, the central projections of different nociceptive neurons innervating the dorsal horn of the spinal cord were not affected in *Kif26a*$^{-/-}$ and control mouse lines at postnatal 1day. These findings suggest that KIF26A negatively regulates the innervation and branching of peripheral but not central projection of DRG sensory neurons.

Next, I investigated the physiological performance of primary cultured DRG neurons on postnatal 15 days by measuring the $[Ca^{2+}]_i$ response challenged by capsaicin, because the ion-channel TRPV1 is believed to be a major sensor of noxious heat. Rapid activation of TRPV1 receptor was studied by monitoring the increase in intracellular calcium concentration $[Ca^{2+}]_i$ following to exposure to capsaicin. I stimulated the neurons with Capsaicin using a perfusion system on the confocal microscopy and measured the time course of $[Ca^{2+}]_i$

elevation using the fluorescence intensity of calcium indicator, Fluo-4, AM. Perfusion on the neurons with MEM containing 1 μM capsaicin resulted in an abrupt elevation of $[Ca^{2+}]_i$ levels similarly in both *Kif26a*$^{-/-}$ and *Kif26a*$^{+/+}$ neurons. After short stimulation with capsaicin, the elevated $[Ca^{2+}]_i$ level rapidly decreased to the basal level in the wild-type neurons after intensive wash with the complete medium. Surprisingly, in the *Kif26a*$^{-/-}$ neurons, the $[Ca^{2+}]_i$ elevation was persisted significantly. This cellular abnormality of *Kif26a*$^{-/-}$ neurons can explain the hyperalgesia at the individual level.

As a molecular basis of these elevated and persistent calcium responses in *Kif26a*$^{-/-}$ DRG neurons, I respectively conducted phosphorylation assays of TRPV1 and PMCA proteins. First, it has been reported that expression and phosphorylation levels of TRPV1 regulate the sensitivity of TRPV1 responsible for the sensation of noxious heat stimuli. The protein amount and phosphorylation level of TRPV1 were compared in DRG neurons of *Kif26a*$^{-/-}$ and *Kif26a*$^{+/+}$ 15-day-old mice. The expression levels of TRPV1 and pTRPV1 were compared after the amount of samples was normalized by the amount of KIF5A by immunoblotting. As a result, I found that the amount of TRPV1 was slightly increased in *Kif26a*$^{-/-}$ DRGs while phosphorylation levels of TRPV1 was significantly increased in *Kif26a*$^{-/-}$ DRGs. This could explain the molecular mechanism for enhanced capsaicin response of DRG neurons. Second, the plasma membrane Ca^{2+} ATPase (PMCA) provides the predominant mechanism for extrusion of Ca^{2+} from cytoplasm of DRG neurons. Because previous studies demonstrated that tyrosine phosphorylation of PMCA inhibits pump activity, I sought to biochemically investigate the tyrosine phosphorylation level of PMCA in KIF26A-knockdown neurons. A *Kif26a* miRNA vector could successfully knockdown KIF26A in F11 cells, compared with a scrambled miRNA vector as a negative control. Then, PMCA was immonoprecipitated from KIF26A-knockdown F11 cells and labeled with anti-phosphotyrosine antibody. As a result, the knockdown cells significantly elevated tyrosine phosphorylated levels of PMCA compared with the negative control suggesting that PMCA was inactivated by KIF26A deficiency. These data will explain the persistent $[Ca^{2+}]_i$ elevation after withdrawal of capsaicin stimulation in *Kif26a*$^{-/-}$ DRG neurons.

Previous studies reported that PI3K and MAPK pathways could be activated by capsaicin in various types of cells including DRG neurons. Therefore, responsiveness of DRG neurons to the capsaicin stimulation could be tested by observing the phosphorylation level of Akt and Erk1/2. Accordingly, the activation of PI3K and MAPK signaling pathways were tested using an anti-phospho-Akt (pAkt) and an anti-phospho-Erk1/2 (pErk) antibodies respectively. The cultured DRG neurons were stimulated with capsaicin, with or without extensive washout of capsaicin, fixed and subjected to immunofluorescence staining against pAkt and pErk1/2. As a result,

the fluorescence intensities of pAkt and pErk1/2 in Substance-P^+ *Kif26a*$^{+/+}$ neurons significantly decreased after capsaicin washout. pAkt and pErk1/2 levels of *Kif26a*$^{-/-}$ neurons were already higher than that of *Kif26a*$^{+/+}$ neurons at the capsaicin activation phase. Furthermore, they were significantly failed in inactivation of these signaling pathways after capsaicin washout. These results demonstrated that PI3K and MAPK signaling pathways of *Kif26a*$^{-/-}$ DRG neurons were hypersensitive and persistently activated by capsaicin stimulation.

I have further investigated whether *Kif26a*$^{-/-}$ neurons were hypersensive in NGF/TrkA-mediated MAPK and PI3K stimulation, because NGF has been demonstrated as an important extracellular signaling molecule in enhancing the sensation of pain. Injection of NGF could induce thermal and mechanical hyperalgesia in adult rats and result in increased sensitivity to noxious heat and mechanical stimuli in humans. Meanwhile, NGF-TrkA signaling has been well characterized to be essential for the sensitization of TRPV1. The cultured DRG neurons were treated with NGF for and stained using antibodies that recognize pAkt and pErk. As a result, the fluorescent intensities of pAkt and pErk were found significantly stronger in *Kif26a*$^{-/-}$ neurons than those of *Kif26a*$^{+/+}$ neurons. These results suggested that a hyperactivated NGF signaling could also enhance the hyperalgesia of *Kif26a*$^{-/-}$ PANs.

In a previous study from our lab, KIF26A negatively regulated the GDNF-Ret-mediated Grb2-SHC interaction and suppressed the PI3K and MAPK signal transductions in enteric neurons. Because NGF-TrkA and GDNF-Ret signaling share the similar downstream signaling pathways mediated by the SHC-Grb2 complex, I sought to investigate whether KIF26A negatively regulates the MAPK and PI3K signaling via affecting the Grb2-SHC interaction also in the DRG neurons. Immunoprecipitation of a DRG cell line F11 showed that the level of SHC-Grb2 complex formation upon NGF stimulation was significantly increased by KIF26A deficiency. Furthermore, co-immunoprecipitation of endogenous KIF26A and endogenous Grb2 was verified using F11 cell lysates. These results suggested that negative regulation of Grb2 by KIF26A plays an important role in suppressing the MAPK and PI3K signaling also in DRG neurons, thereby properly controlling the noxious sensitivity of the PANs.

In conclusion, my study resolved a new function of kinesin superfamily protein 26A as a regulator of pain sensation and also established a relationship among KIF26A, NGF-TrkA signaling, and calcium regulation in the sensory nervous system through its general role in modulating Grb2 signaling. It provided evidence that the signaling modulatory role of this atypical kinesin can work in general and be responsible for wider range of disease and development than previously expected, which will be available for use in basic research of cell signaling and in development of therapeutic applications.