Graduate School of Advanced Science and Engineering
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博 士 論 文 概 要
Doctoral Thesis Synopsis

論文題目

Thesis Theme

Functional Analysis of Collapsin Response Mediator Protein 4 in Recovery after Neural Injury

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Most of tragic central nervous system (CNS) diseases, such as Alzheimer's disease, Parkinson's disease, and even spinal cord injury (SCI) have lots of similarities in how neurons degenerate. CNS disorders involve immense axonal degeneration and long-lasting secondary damage on nervous system by inflammation. CNS manages to help the cells with molecular defenses, but ultimately killing the cells instead. No more than waiting and watching for the extent of any recovery are done when a spinal cord is bruised or injured. The spinal cord is the main motor and sensory pathway for connecting the brain and peripheral nervous system. The vertebrate spinal cords are divided into 31 different levels. The injuries induce paraplegia below the injury site with lots of malfunctions, such as hemiplegia, excretory disorder and dysfunction of thermoregulation. However, there is no promising treatment for SCI, and molecular findings are thus needed to achieve a maximal recovery.

Axonal outgrowth inhibitors and scar formation are two major obstacles to CNS repair. Although numerous molecular and cellular mechanisms have been found for CNS repair after traumatic injury, all the indicated extrinsic molecules have each problem. For example, *in vivo* studies of myelin-associated inhibitors (MAIs) in loss-of-function still produce opposing results on their role in axon regeneration after CNS trauma. There are concerns of strong side effects when targeting axon guidance molecule semaphorin3A, as embryonic lethality of Sema3A-deficient mice is observed possibly because of its broad expression in neural tissues as well as in other organs. Regions of the enzyme-digested chondroitin sulfate proteoglycan (CSPG) in scar tissues after CNS injury remain rather inhibitory to neurite outgrowth. No target molecule that regulates both axonal growth and scarring has been identified possibly because the field lacks a perspective on neuronal intrinsic mechanism. Thus in order to obtain further regenerative capacity with less side effects outside of nervous system by disrupting these inhibitory signals, this thesis focuses on a common downstream molecule that is highly expressed in the nervous system.

Cytoskeletal dynamics is a key factor limiting the regenerative capacity of the CNS in terms of axon formation, inflammation, and scarring. Collapsin response mediator protein 4 (CRMP4) is one of the CRMP family proteins that are highly expressed in the developing and adult nervous systems among vertebrates and regulates aspects of neurite growth by their binding to the cytoskeleton. Sema3A-induced phosphorylation of CRMP4 (pCRMP4) reduces the CRMP4 binding affinities for tubulin heterodimer and F-actin, resulting in cytoskeletal depolymerization. CRMP4 allele produces short and long isoforms that are different in the length of their N-termini, which have otherwise been referred to as "a" (short) and "b" (long) isoforms, respectively. Previous studies have demonstrated that long-form CRMP4 (CRMP4b) is required for inhibitory responses to MAIs *in vitro*. This suggests a possibility of CRMP4 involvement in CSPG-induced signaling because inhibitory signals by MAIs and CSPG share intracellular mechanisms through their common receptor NgR. Moreover, injury-induced neuronal calpain activation produces a C-terminus-truncated form of CRMP4 (tCRMP4) that initiates neuronal cell death. CRMP4 is therefore a common mediator of several inhibitory signaling pathways operating after traumatic injury.

However, it remains largely unknown whether the involvement of CRMP4 in myelin-associated glycoprotein (MAG)-induced axonal protective effects and how CRMP4 functions following a traumatic CNS injury *in vivo*. The goal of this thesis is to clarify the function of CRMP4 in recovery after neural injury by CRMP4-deficient (*Crmp4*−/−) mouse model.

This thesis consists of five chapters, which are summarized as follows.

In chapter 1, I described the scientific background and related researches and the reasons why I focus on Collapsin response mediator protein 4 (CRMP4) as described above.

In chapter 2, I described the experimental materials and methods.

Chapter 3 can be divided into two parts, my results from an *in vitro* study and an *in vivo* study.

First, I described the analyses of the role of CRMP4 in MAG-induced signaling pathways *in vitro* using dorsal root ganglion (DRG) neuronal culture. I showed the involvement of CRMP4 both in MAG-induced axonal outgrowth inhibition and protection against acute toxic insult.

To determine the role of CRMP4 on those points, I took advantage of *Crmp4*−/− mice where CRMP4 protein expression was specifically and totally eliminated. To confirm the involvement of CRMP4 in axonal inhibitory response, DRG neurons derived from *Crmp4*−/− mice and *Crmp4*+/+ control mice were stimulated with soluble MAG-Fc. While growth cones from the *Crmp4*+/+ DRG neurons were collapsed by MAG-Fc, the ratio of MAG-Fc-mediated growth cone collapse was reduced in the *Crmp4*−/− DRG neurons. This result clearly confirmed that CRMP4 is required for MAG-induced inhibition of axon growth and collapse of growth cone. Next, to determine the role of CRMP4 in axonal viability after acute toxic insult, I administrated vincristine (VNC), a inhibitor of microtubule assembly, into the cultured *Crmp4*+/+ and *Crmp4*−/− DRG neurons. Deletion of CRMP4 enhanced vulnerability to VNC in cultured DRG neurons. Finally, to examine the involvement of CRMP4 in MAG-induced axonal protection against acute toxic insult, cultured *Crmp4*+/+ and *Crmp4*−/− DRG explants were co-incubated with 25 μg/mL MAG-Fc and 20 nM VNC. While the suppression of VNC-induced axonal degeneration with MAG was observed in *Crmp4*+/+ DRG explants, *Crmp4*−/− DRG axons were almost completely degenerated with VNC treatment regardless of MAG-Fc presence, suggesting that CRMP4 is required for MAG-mediated axonal protection.

In the latter part of Chapter 3, I examined the role of CRMP4 in recovery from spinal cord injury *in vivo*. Enhanced regenerative responses by neuroprotection and limited scar formation were observed upon CRMP4 deletion.

In vitro studies have shown the dual role of CRMP4 in MAG-induced signals, but the significance of *Crmp4* deletion after CNS injury *in vivo* has never been examined. Therefore, I next examined the role of CRMP4 in regenerative and/or degenerative responses after CNS traumatic injury *in vivo*. I utilized a 1.5-mm-depth near-complete dorsal transection model to sever the whole gray matter of the spinal cord and a group of defined pathways, including raphe-spinal and cortico-spinal tract (CST) axons and all their branches, leading to hindlimb motor dysfunction. The hypothesis that CRMP4 contributes to the

limitation of recovery after adult CNS trauma is supported by several major findings from this study on *Crmp4*−/− mice. First, the expression levels of pCRMP4, tCRMP4, and CRMP4b, which are suggested to contribute considerably to limiting axonal growth and to promoting cell death, are significantly increased at the lesion site in spinal cord. Second, increased CRMP4 expression in activated microglia/macrophages and reactive astrocytes might contribute to secondary injury, including inflammation and scarring after spinal cord lesion. Third, the deletion of CRMP4 would have neuroprotective effects including preservation of microtubule polymerization, cell survival, delayed demyelination, and tissue sparing. This leads to axonal growth and locomotor recovery after SCI.
In chapter 4 to 5, the results of this thesis were discussed and future prospects were summarized.

In vitro studies demonstrate that CRMP4 mediates contradictory signaling pathways; inhibition of axon growth and axonal protection from toxic insult. It is possible that different forms of CRMP4 would mediate each signal. CRMP4b was dramatically upregulated after *in vivo* injury, whereas substantial change of CRMP4a level was not found. The data that deletion of CRMP4 enhanced regenerative responses after SCI implicates that inhibitory roles of CRMP4b (possibly, pCRMP4 and tCRMP4 also) might be dominant *in vivo* CNS traumas, however, this is yet to be proven. To examine which form of CRMP4 plays the represent roles, transgenic lines for conditional gene mutation could be effective. Alternative, for instance, *in vivo* delivery of a peptide that expresses the unique N-terminal domain of CRMP4b (C4RIP– CRMP4b–RhoA inhibitory peptide) might be used to assess the role of CRMP4b reduction after CNS injury *in vivo*.

Additionally, it is still unclear which is rather beneficial, the reduction in inflammation and scarring or enhanced axon viability to regenerative responses in *Crmp4*−/− spinal cords. Conditional *Crmp4* depletion in astroglia and/or microglia may be worth trying to clarify that point. Furthermore, the molecular basis of CRMP4 in inflammatory cells remains largely unknown. Recently a study has shown that CRMP4 binds to F-actin in activated *in vitro* microglial model cell and promotes its migratory property. However, which signaling pathways upregulate CRMP4 expression in reactive astrocytes and/or activated microglia/macrophages has never been clearly shown. It is conceivable that CNTF/JAK/ STAT pathway upregulates CRMP4 after neural injury. To determine that, *in vitro* studies using cultured astrocytes and murine BV2 microglia could be carried out.

Although additional studies for further detail signaling pathways are needed, my data suggest the therapeutic potential of CRMP4 with minimal side effects: (i) the inhibitory and toxic forms of CRMP4 were specifically increased in injured spinal cord, and (ii) I observed no gross abnormalities in *Crmp4*−/− mice due to a possible redundancy among CRMP family proteins. Neurological diseases are frequently associated with axonal degeneration and secondary tissue damage by inflammation. The concept in this thesis that a crucial factor controls both neuronal and glial responses could be applicable for therapeutic strategies for other diseases in the nervous system. In conclusion, the present study demonstrates that deletion of a single protein—CRMP4—resulted in the reduction of axonal outgrowth inhibition in neurons, inflammatory responses of glia, and scarring responses of glia, thereby promoting axonal growth and functional recovery after SCI. CRMP4 may be a possible therapeutic target for the treatment of human patients with SCI.

早稲田大学　博士（理学）　学位申請　研究業績書

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	Journal articles
論文○	1) **Nagai, J.**, Kitamura, Y., Owada, K., Yamashita, N., Takei, K., Goshima, Y., Ohshima, T. *Crmp4* deletion promotes recovery from spinal cord injury by neuroprotection and limited scar formation. *Sci. Rep*., (2015) 5:8269
論文○	2) **Nagai, J.**, Goshima, Y., Ohshima, T. CRMP4 mediates MAG-induced inhibition of axonal outgrowth and protection against Vincristine-induced axonal degeneration. *Neurosci. Lett.*, (2012) 519: 56-61.
論文	3) Niisato, E., **Nagai, J.**, Yamashita, N., Abe, T., Kiyonari, H., Goshima, Y., Ohshima, T. CRMP4 suppresses apical dendrite bifurcation of CA1 pyramidal neurons in the mouse hippocampus. *Dev. Neurobiol.*, (2012) 72(11): 1447-57.
論文	4) Niisato, E., **Nagai, J.**, Yamashita, N., Nakamura, F., Goshima, Y., Ohshima, T. Phosphorylation of CRMP2 is involved in proper bifurcation of the apical dendrite of hippocampal CA1 pyramicdal neurons. *Dev. Neurobiol.*, (2013) 73(2): 142-51.
	Conferences
講演	1) **Jun Nagai**, Yoshiteru Kitamura, Kazuki Owada, Yoshio Goshima, Toshio Ohshima. Genetic modifications of Crmp enhance axonal regrowth after spinal cord injury by reducing cytoskeletal destabilization and inflammatory responses. The 37th Annual Meeting of the Molecular Biology Society of Japan. 2014. Yokohama, Japan. (Poster)
講演	2) **Jun Nagai**, Yoshiteru Kitamura, Kazuki Owada, Yoshio Goshima, Toshio Ohshima. Genetic modifications of Crmp enhance axonal regrowth after spinal cord injury by reducing cytoskeletal destabilization and inflammatory responses. SfN Annual Meeting “Neuroscience 2014”. Nov. 2014. Washington D.C., USA. (Poster)

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種 類 別	題名、　発表・発行掲載誌名、　発表・発行年月、　連名者（申請者含む）
講演	3) **Jun Nagai**. The roles of axon guidance molecules CRMPs in regeneration after CNS injury. Associates of Young Researchers of Physiology Forum. Jul. 26th 2014. Tokyo, Japan. (Oral Presentation)
講演	4) **Jun Nagai**, Yoshiteru Kitamura, Naoya Yamashita, Kohtaro Takei, Yoshio Goshima, Toshio Ohshima. Loss of CRMP4 promotes axonal regrowth and recovery after spinal cord injury The 6th HOPE meeting Mar. 2014. (Poster)
講演	5) **Jun Nagai**, Yoshiteru Kitamura, Naoya Yamashita, Kohtaro Takei, Yoshio Goshima, Toshio Ohshima. The role of CRMP in axonal regrowth after spinal cord injury. SfN Annual Meeting “Neuroscience 2013”. Nov. 2013. San Deigo, CA, USA. (Poster)
講演	6) **Jun Nagai**, Yoshiteru Kitamura, Naoya Yamashita, Yoshio Goshima, Toshio Ohshima. The roles of CRMP in axonal sprouting and recovery after spinal cord injury. Neuro2013. P2-2-69. Jun. 2013. Kyoto, Japan. (Poster)
講演	7) **Jun Nagai,** Kodai Sasamoto and Toshio Ohshima. The role of Cdk5 kinase activity in GABAergic interneurons in forebrain. The 6th Neurodevelopmental Symposium. Mar. 14th 2013. Saitama, Japan. (Poster)
講演	8) **J. Nagai**, Y. Kitamura, Y. Goshima, T. Ohshima. The role of Collapsin mediator response protein 4 in axonal regeneration and degeneration. The 35th Annual Meeting of the Molecular Biology Society of Japan. Dec. 2012. Fukuoka, Japan. (Poster)
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