論文の内容の要旨

論文題目

Roles of P/Q-type Ca^{2+} channel and Arc in activity-dependent climbing fiber to Purkinje cell synapse elimination in developing cerebellum

（発達期小脳の活動依存的な登上線維－プルキンエ細胞シナプスの刈り込みにおける P/Q 型カルシウムチャネルと Arc の役割）

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<Introduction>

Elucidating how functional neural circuits are constructed during postnatal development is a fundamental issue to the understanding of brain functions. Neural circuit formation during postnatal development involves a process known as synapse elimination. In this process, necessary synapses are selectively strengthened and redundant connections are removed in early postnatal life. The climbing fiber (CF) to Purkinje cell (PC) synapse in the cerebellum provides an excellent model to study the cellular and molecular mechanisms of synapse elimination in the developing brain. Previous reports show that decreasing PC activity in mice by either overexpression of chloride channels or deletion of P/Q-type voltage-dependent Ca^{2+} channel (VDCC) in PC-selective manners impairs CF synapse elimination, but how PC activity determines CF synapse elimination still remains unclear.

Arc (also known as *Arg3.1*) is a well-known immediate early gene, acting as an effecter protein downstream of multiple neuronal signaling pathways. The function of Arc has been characterized in the hippocampus and cerebral cortex as to its role in synaptic plasticity, homeostatic plasticity and experience-dependent plasticity in the remodeling of neocortical circuits. Recently Arc was shown to be required for the late-phase long-term depression (LTD) in cultured cerebellar PCs. However, the role of Arc in developing cerebellar circuits has not been explored. In the present study, I aimed to disclose the interplay between PC activity, Arc and CF synapse elimination.

<Results>

I newly developed an *in vitro* olivo-cerebellar coculture preparation which enables us to perform pharmacological, genetic and optogenetic manipulations in fast and reproducible manners. The olivo-cerebellar coculture consists of a parasagittal cerebellar slice from mice at postnatal day 10 (P10) and an explant of the medulla oblongata containing the inferior olive obtained from rats at embryonic day 15. The cocultures well mimic *in vivo* cerebellar

circuits and reproduce CF synapse formation and elimination with similar molecular mechanisms to those *in vivo*. More than 60 % of PCs are innervated by three or more CFs at 7 days *in vitro* (DIV). CF synapse elimination reaches a plateau at 15 DIV, with about 70 % of PCs innervated by one or two CFs.

Using this coculture preparation, I examined the effect of increasing PC activity on CF synapse elimination. To increase PC activity, I expressed channelrhodopsin-2 (ChR2) in PCs under the PC-specific L7 promoter by using a lentiviral gene transfer technique. I applied 2-day photostimulation to cocultures from 10 or 11 DIV, when redundant climbing fibers are being eliminated. After the 2-day photostimulation, I found that CF synapse elimination was accelerated in ChR2-expressing PCs when compared with uninfected (control) PCs in the same slices.

Previous studies demonstrate that P/Q-type VDCC in PCs is crucial for CF synapse elimination in developing cerebellum. P/Q-type VDCC is the major high-threshold VDCC in PCs and is considered to be activated when PCs are depolarized. Thus, I hypothesized the acceleration of CF synapse elimination by ChR2-mediated elevation of PC activity was caused by the increase of Ca^{2+} influx through P/Q-type VDCC. I examined the effect of knockdown of P/Q-type VDCC (P/Q knockdown) on the acceleration of CF synapse elimination by comparing the CF innervation patterns in PCs with (1) ChR2 expression, (2) P/Q knockdown + ChR2 expression and (3) P/Q knockdown + EGFP expression. I applied the 2-day blue light illumination to these three groups of coculture from 10 or 11 DIV. I found that significantly higher number of CFs innervated PCs with P/Q knockdown + ChR2 expression when compared to those with ChR2 expression. On the other hand, significantly higher number of CFs innervated PCs with P/Q knockdown + EGFP expression when compared to those with P/Q knockdown + ChR2 expression. These findings demonstrate that the acceleration of CF synapse elimination by the 2-day excitation of PCs is significantly blocked by P/Q knockdown. Thus, Ca^{2+} influx through P/Q-type VDCC is an important factor for the acceleration of CF synapse elimination.

Neural activity induces a number of Ca^{2+}-dependent genes involved in synapse development, maturation and refinement. Previous studies demonstrate that CF synapse elimination is an activity-dependent process mediated by P/Q-type VDCC. Thus, I examined the possibility that some Ca^{2+}-dependent genes regulate CF synapse elimination. Expression of Arc is tightly coupled to neural activity downstream of multiple signaling pathways, including Ca^{2+} influx through VDCC. Arc mRNA is detectable in PCs in mouse cerebellum at P4, and its expression increases thereafter during postnatal development (Allen Brain Atlas). I compared Arc mRNA expression level in the mouse cerebellum at P9 and P16 by real-time PCR. Arc mRNA expression level at P16 was significantly higher than that at P9, indicating that the expression of Arc increases during the period of CF synapse elimination.

To examine whether the Arc expression in PCs is activity-dependent, I used Arc-pro-Venus-pest Tg mice in which Venus fluorescent reporter is expressed under the control of Arc promoter. I constructed cocultures of cerebellar slices derived from the Arc-pro-Venus-pest Tg mice and explants of medulla oblongata from rats. Robust expression of Arc was observed mainly in PCs by either membrane depolarization (high K^+) or blockade of GABAergic inhibition (picrotoxin). The increase in Arc expression was suppressed when P/Q-type VDCC blocker (ω-agatoxin IVA) was applied in the high K^+ or picrotoxin-containing culture medium. Similar suppression of high K^+ or picrotoxin-induced elevation of Arc expression was observed in cocultures with PC-specific knockdown of

P/Q-type VDCC. These results indicate that Arc is expressed in PCs in an activity-dependent manner, which requires the activation of P/Q-type VDCCs in PCs.

To test the role of Arc in CF synapse elimination, I constructed lentiviruses expressing engineered miRNA directed against Arc (Arc miRNA) together with EGFP under L7 promoter. I found significantly higher number of CFs innervated PCs with Arc miRNA-expression at 15-17 DIV, indicating that Arc is involved in CF synapse elimination in cocultures.

P/Q-type VDCC is required for the acceleration of CF synapse elimination caused by increasing PC activity. Arc is expressed in PC in an activity-dependent manner, which requires the activation of P/Q-type VDCC in PC. Therefore, it is legitimate to assume that Arc is necessary for the acceleration of CF synapse elimination. To test this possibility, I examined the effect of Arc knockdown on the acceleration of CF synapse elimination by comparing the CF innervation patterns in PCs with (1) ChR2 expression, (2) Arc knockdown + ChR2 expression and (3) Arc knockdown + EGFP expression. I found that significantly higher number of CFs innervated PCs with Arc knockdown + ChR2 expression when compared to those with ChR2 expression. On the other hand, significantly higher number of CFs innervated PCs with Arc knockdown + EGFP expression when compared to those with Arc knockdown + ChR2 expression. These findings indicate that the acceleration of CF synapse elimination by the 2-day excitation of PCs is significantly blocked by Arc knockdown. Together with the observations that Arc is tightly coupled with PC activity, these results suggest that activity-dependent expression of Arc is a key step to the acceleration of CF synapse elimination.

To confirm the role of Arc in CF synapse elimination *in vivo*, I injected lentiviruses expressing Arc miRNA together with EGFP under L7 promoter into the mouse cerebellar vermis at P2-3. The cerebella were examined by electrophysiological analyses at P19-26, when most PCs have become innervated by single CFs in wild-type mice. I examined the CF innervation pattern in virus-infected (Arc knockdown) and uninfected (control) PCs by using whole-cell recordings from PCs in acute cerebellar slices. I found that the regression of surplus CFs was impaired in PCs with Arc knockdown, demonstrating that Arc plays a pivotal role in CF synapse elimination *in vivo*.

<Discussion>

Optogenetics in cocultures is an effective method to manipulate activities of neural circuits because *in vitro* preparations can be photostimulated directly and reliably. In the present study, I applied optogenetics to the study of activity-dependent CF synapse elimination in olivo-cerebellar cocultures and demonstrated the crucial role of postsynaptic PC activity in CF synapse elimination. However, my results do not exclude possible roles of CF's presynaptic activity or activities of elements other than CF-PC synapse such as parallel fiber (PF) inputs, inhibitory inputs or influence from Bergmann glia. Combined use of optical excitation (ChR2), red-shifted optical excitation (C1V1) and/or optical inhibition (halorhodopsin) in olivo-cerebellar cocultures to differentially manipulate activities of PCs and olivary neurons may elucidate roles of CF's presynaptic activity and interaction between pre- and postsynaptic activities in CF synapse elimination. Furthermore, cell-type specific expression of these molecules in granule cells (origins of PF inputs) or inhibitory interneurons and manipulation of their activities are useful for elucidating contribution of PF inputs and inhibitory inputs to CF synapse elimination.

I elucidated the involvement of P/Q-type VDCC and Arc in the acceleration of CF synapse elimination caused by the elevation of PC activity. However, since the acceleration was not abolished completely by the knockdown of P/Q-type VDCC or Arc, other factors may contribute to the acceleration of CF synapse elimination. The candidates include Ca^{2+} influx through other types of VDCCs such as L-type, Ca^{2+} influx directly through the cation channel of ChR2, and other activity-dependent transcribed genes.

How Arc is involved in CF synapse elimination remains to be examined. Previous studies in the hippocampus indicate that Arc plays an important role in trafficking of AMPA-type glutamate receptors (AMPARs). It is unknown whether Arc is involved in AMPAR endocytosis in PCs. However, my present results that the amplitude of CF-EPSC in Arc knockdown PCs was larger than control PCs suggest that Arc plays a role in AMPAR endocytosis also in PCs. It is possible that AMPA receptor endocytosis may contribute to the weakening of redundant CF synaptic inputs and the eventual elimination of such CFs. Defect of this process in Arc knockdown PCs may lead to impaired CF synapse elimination.

Disordered expression of Arc has recently been reported in neurodevelopmental diseases such as Fragile X syndrome and Angelman syndrome. Since my present study shows importance of Arc in synapse elimination in developing cerebellum, it is possible that some symptoms in these diseases might be related to abnormality of neural circuit organization and function. It is important to examine whether and how Arc contributes to neural circuit formation and refinement in brain regions that are considered to be relevant to the symptoms.