博士学位論文審査要旨

２０１６年1月２２日

論文題目：Critical Role of Ca^{2+} Current Facilitation in the Short-Term Facilitation of Purkinje Cell – Purkinje Cell Synapses (プルキンエ細胞間シナプス短期促通現象における Ca 電流の役割)

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要　旨：

本論文では、脳高次機能の発現に極めて重要な役割を果たすシナプス可塑性のメカニズムに関して、提出者が新たに発見した知見をもとに論じている。哺乳類の運動を司る小脳には、小脳内で統合された情報を出力するプルキンエ細胞(PC)が存在する。PC は、深部小脳核(DCN)に軸索を投射しており、このシナプスは発火が連続すると短期シナプス抑圧を呈する。一方、PC は近接した PC にも軸索を投射しシナプスを形成するが、PC-PC 間シナプスは短期シナプス促通の性質を持つ。PC-PC 間シナプスにおいてシナプス促通が惹起されるメカニズムや、PC がシナプス投射先によって異なるシナプス可塑性を呈する分子機構は不明であった。

このような背景の下、提出者はラット小脳由来の神経初代培養細胞系を活用して、従来は困難であったシナプス終末からの直接パッチクランプ記録に成功し、PC-PC 間シナプスにおける短期シナプス促通のメカニズムを解明した。まず、PC に投射した PC シナプス終末にパッチクランプ電極を通じて活動電位様の連発刺激を与えると、Ca^{2+}依存的な Ca^{2+}電流の促進が見られた。また、連発刺激の間隔を変化させると、シナプス終末における Ca^{2+}電流変化の約 4.5 乗に比例したシナプス後電流の変化が観察された。一方、Ca^{2+}電流の促進が起こらないように活動電位強度を設定した連発刺激を与えても、シナプス促通は見られなかった。このことから、PC-PC 間シナプスで見られるシナプス促通は Ca^{2+}電流の促進によって惹起されると結論された。一方、シナプス抑圧を呈する PC-DCN でも、シナプス終末に直接連発刺激を与えると、PC-PC シナプスと同様の Ca^{2+}電流促進とシナプス促通が観察された。最近、所属研究室から PC-DCN でのシナプス抑圧がシナプス終末近傍における活動電位の減衰に起因することが報告されたが(Kawaguchi and Sakaba, 2015)、提出者による新たな知見は、投射先特異的なシナプス可塑性の発現における活動電位伝導効率の重要性を裏付けるものである。これらの研究結果は、生理学分野で有力な国際紙である Journal of Physiology　593 号（2015 年）に筆頭著者として発表された。

以上、本論文は、脳機能を支えるシナプス可塑性のメカニズムの一端を解明したものであり、実施された革新的な技術はシナプス可塑性研究の更なる進展を期待させるものである。よって、本論文は、博士（理学）（同志社大学）の学位を授与するにふさわしいものであると認められる。

総合試験結果の要旨

２０１６年1月２２日

論 文 題 目： Critical Role of Ca^{2+} Current Facilitation in the Short-Term Facilitation of Purkinje Cell – Purkinje Cell Synapses (プルキンエ細胞間シナプス短期促通現象における Ca 電流の役割)

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要　　旨：

博士論文提出者は、2013 年 4 月に本学大学院脳科学研究科発達加齢脳専攻一貫制博士課程に第 3 年次転入学し、現在、在籍中である。主に、神経初代培養細胞を用いた電気生理学的測定に習熟し、脳内におけるシグナル伝達の場であるシナプスの生理学に関する知識の習得、研究に研鑽した。本博士論文の内容は、Journal of Physiology　593 号（2015 年）に筆頭著者として既に掲載された。

2016 年 1 月 20 日午後 1 時より約 1 時間半にわたり提出論文に関する審査会を行ない、提出者による英語でのプレゼンテーションと質疑応答を行なった。プレゼンテーションでは、研究の目的、方法、結果、結論、考察が適切に述べられ、質疑応答に関しても提出者の説明により十分な理解が得られたと考える。

更に審査会終了後、審査委員により論文内容ならびにこれらに関連する脳科学分野の諸問題について非公開の口頭試問を実施した結果、本論文提出者は研究者として十分な学力と、国際的に活躍するための語学力（英語）を有していることが認められた。

よって、総合試験の結果は合格であると認める。

博 士 学 位 論 文 要 旨

論 文 題 目 : Critical Role of Ca^{2+} Current Facilitation in the Short-Term Facilitation of Purkinje Cell – Purkinje Cell Synapses
プルキンエ細胞間シナプス短期促通現象における Ca 電流の役割

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要　　旨 :
Short-term presynaptic plasticity (STF) is a phenomenon in which repeated stimulation of the presynaptic terminal leads to a transitory increase in the amount of neurotransmitter (NT) released by the presynaptic cell. STF plays a critical role in neural information processing; yet, despite its importance, the mechanism underlying it remains unclear. Several hypotheses have been proposed to explain this process: temporal summation of residual Ca^{2+}, modulation of action potential (AP) waveform, saturation of Ca^{2+} buffer proteins, facilitation of Ca^{2+} current (I_{Ca}^{2+}) through voltage-gated Ca^{2+} channels, rapid recruitment of releasable vesicles to the active zone and Ca^{2+}-dependent positive modulation of vesicle release machinery.
The study of STF has been hindered by the small size of presynaptic terminals. For a correct and accurate assessment of the mechanisms underlying the short-term facilitation phenomenon at presynaptic terminals, direct access to the terminal is often required. Patch-clamping of synaptic terminals in brain slices represents a difficult task for most synapses. Recordings from the cell's soma may not be able to dissect the effects taking place at synaptic terminal level, and current imaging techniques may not provide the required spatiotemporal resolution to analyze the biophysical properties involved in the NT release processes. To address the issue of the difficulties of patch-clamping from presynaptic terminals, we attempted to clarify the mechanism mediating STF at synapses between cerebellar Purkinje cells (PCs) using dissociated rat cerebellar cultures. In our culture preparations, access to the synaptic terminals is relatively easier than in cerebellar slice, owning the increased visibility to reduced surrounding tissue and the easiness of virus transfection.
By infection with adeno-associated virus (AAV) vector carrying enhanced green fluorescent protein (EGFP) under the control of CAG promoter (AAV-CAG-EGFP), we could visually identify PCs and their synaptic terminals through fluorescence. Pair recordings of synaptically connected PCs showed STF of synaptic transmission upon paired-pulse stimulation, similar to that observed in slices.
To study the mechanism underlying short-term facilitation at PC–PC synapses, we performed direct whole-cell voltage patch-clamp recordings from a presynaptic PC terminal impinging on the soma or proximal dendrite of a PC, and the impinged postsynaptic PC. We recorded the presynaptic Ca^{2+} current (I_{Ca}^{2+}) upon single stimulation and the resultant postsynaptic current (PSC). To mimic the physiological conditions of synaptic transmission, in which a presynaptic terminal is activated by the arrival of an AP, stimulation was done by applying a voltage command with an action potential (AP) waveform that was previously

recorded from a PC terminal. To study the relationship between the stimulating AP command, the presynaptic Ca^{2+} currents and the PSCs, we gradually changed the peak amplitude of the AP command. As it became larger in amplitude, the Ca^{2+} current almost linearly increased. On the other hand, the PSC amplitude changed supralinearly, and showed a 4–5th power dependency on the Ca^{2+} current amplitude. This supralinearity is similar to that observed in the relationship between Ca^{2+} concentration in the presynaptic terminal ($[Ca^{2+}]_i$) and the amount of neurotransmitter released, which suggests that the facilitation of the PC – PC synapse may be primarily due to presynaptic processes.

Following this, we stimulated the presynaptic terminal with a pair of AP-like waveforms, and observed that the presynaptic Ca^{2+} current ($I_{Ca^{2+}}$) was facilitated depending on the intracellular Ca^{2+} concentration. Application of the Ca^{2+} chelator EGTA accelerated the decay of the STF observed, but did not suppress the facilitation of $I_{Ca^{2+}}$. However, application of BAPTA, a Ca^{2+} chelator with similar Ca^{2+} affinity (Kd) as EGTA but with much faster Ca^{2+} binding kinetics, abolished it. These results suggest that the facilitation of $I_{Ca^{2+}}$ is tightly coupled with Ca^{2+} influx.

Taking into consideration that transmitter release is supra-linearly dependent on the $[Ca^{2+}]_i$ (Fig. 6C), the PPF of Ca^{2+} currents might be a powerful mechanism leading to the STF of synaptic transmission. To study the quantitative relationship between the PPF of Ca^{2+} currents and that of synaptic transmission, we performed paired recordings from a presynaptic PC axon terminal and a postsynaptic PC soma. Paired-pulse AP waveforms with different intervals caused facilitation in both $I_{Ca^{2+}}$ and PSCs. The facilitation of PSCs could be fitted to the 4–5th power of that of $I_{Ca^{2+}}$.

Though the previous results show a strong correlation between PSCs and $I_{Ca^{2+}}$, it does not imply causation. To examine the causal relationship between the PPF of $I_{Ca^{2+}}$ and that of PSC, it was necessary to study the PSCs in the absence of $I_{Ca^{2+}}$ facilitation, and observe whether the PPF of PSCs remained unchanged. For this, following the previous results that show linear dependency of the $I_{Ca^{2+}}$ amplitude and the AP voltage command amplitude, in PC – PC pair recordings during paired stimulation protocol, we next varied the amplitude of $I_{Ca^{2+}}$ during the second AP voltage command by systematically altering the peak amplitude of the second AP (to 1.05, 1, 0.95, 0.9 or 0.85 times the first AP). As the amplitude of the second AP command decreased, that of the second $I_{Ca^{2+}}$ peak was also decreased. As a result of the decreased $I_{Ca^{2+}}$, STF of PSCs disappeared. Interestingly, the point in which STF of PSCs disappear is the same as the point in which STF of $I_{Ca^{2+}}$ also disappear. This is, if the facilitation of $I_{Ca^{2+}}$ is abolished, so will the facilitation of PSCs be abolished. These results strongly suggest that the Ca^{2+}-dependent facilitation of the presynaptic $I_{Ca^{2+}}$ fully mediates the synaptic facilitation observed at the PC-PC synapse.

The results so far precluded major contribution of other mechanisms of facilitation, such as the residual Ca^{2+} hypothesis, which suggested that temporal summation of $[Ca^{2+}]_i$ during paired AP arrivals increases the transmitter release probability. To test this issue further, we first selected PCs that did not expressed EGFP and performed Ca^{2+} imaging using Ca^{2+} sensitive fluorescence indicators. PC synaptic terminals showed very little increase in the fluorescence upon single stimulation, corresponding to an ~10–20 nM of $[Ca^{2+}]_i$ increase. The residual Ca^{2+} increase by 20 nM is estimated to increase the transmitter release by ~1% assuming 4–5th power Ca^{2+} dependence. Thus, in line with the idea that the PPF of the PSCs is predominantly mediated by that of Ca^{2+} currents, the temporal summation of

residual Ca^{2+} probably plays no major role in the PPF of synaptic transmission at PC–PC synapses.

It is interesting to note the contrast between the facilitating synapse between PCs and the depressing synapses between PCs and DCN neurons, the main targets of PCs. PC synapses on a DCN neuron show depression upon high-frequency stimulation due to AP attenuation around synaptic terminals. To study the mechanism underlying the target-dependent opposite forms of short-term synaptic plasticity, we next examined whether the facilitation of Ca^{2+} currents and the resultant enhancement of transmitter release is a unique mechanism of PC synapses on another PC but not on DCN neurons. Our results showed that the Ca^{2+}-dependent facilitation of Ca^{2+} currents causing short-term facilitation is a common feature in PC axon terminals, irrespective of the target neuron type, as long as identical APs are elicited. In reality, AP conduction failures lead to synaptic depression at the synapse between the PC and the DCN neuron.

To summarize our results, we conclude that PC–PC synapses show short-term facilitation of transmitter release predominantly depending on the Ca^{2+}-dependent Ca^{2+} current facilitation. Our data further indicated that the synaptic facilitating property relying on the Ca^{2+} current facilitation was common at PC output synapses, irrespective of the target cells, when identical paired AP commands were applied to the terminal. Therefore, the propagation of action potentials to the terminal determines short-term depression at the synapses between a PC and the neuron in the DCN.